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Differential gene expression and morphological variation in tequila bats (*Leptonycteris yerbabuenae*): adaptations to migration

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Abstract

The study of migration has mainly focused on understanding the patterns and routes that animals follow, and how these movements affect the genetic composition of populations. Studies on the morphology of wings coupled with predictions of aerodynamic theory have shown that even the slightest differences in the shape of wings can have a significant effect on the foraging behaviour and flying ability of bats. More recently, genomic studies opened the door to understand the genetic makeup responsible for triggering and controlling the behaviour. In this thesis, I used genetics to test if the tequila bat (*Leptonycteris yerbabuenae*) is maintaining matrilineal gene flow across its range. Using mtDNA I show that the populations of southern, central and northern Mexico belong to the same species and do not show clear patterns of population structuring but that two haplotype groups may exist. Gene flow is maintained across its range with differences in migratory behaviour observed between the northern and southern populations. My analysis on wing morphology shows that the migratory population has wings with higher aspect ratio and more pointed wing tips that allows them to sustain long-distance flight better than the resident populations; in contrast, the non-migratory populations show morphological adaptations to manoeuver better in a more cluttered environment including a shorter humerus, lower aspect ratio and wing loading. By looking at differences in gene expression I was able to detect potential genes influencing the migratory behaviour. Migratory bats appear to have genetic potential to build more synaptic connections that may allow them to store new information from the environment and show higher expression of genes associated with exploration behaviour compared with resident populations; furthermore, I show that circadian clock genes, influenced by the environment, may contribute to the regulation of the expression of migration. My results on the gene expression of blood show that migratory bats and have a high immune tolerance toward pathogens explained by a diversity of MHC complexes, natural cell killers, antiviral interferons and other immune regulatory elements, traits that could explain how these animals stay healthy despite the inherently challenging task of migrating and moving to new areas potentially containing novel pathogens. This project opens the door to future studies on the mechanisms controlling migration in bats and offers a novel way to understand how genes and the environment paved the way for the evolution of migration, and how phenotypic plasticity continues to shape these organisms.

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Finally, I would like to acknowledge my family and friends who have always been there for me, in particular Ricardo Saldana-Meyer, my husband and the biggest supporter of my work. Thank you, my love, for making things easier for me, for your infinite love and kindness, even when an ocean was between us I always felt you were close to me.

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Author's Declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

Dr. Rodrigo Medellin from the National University of Mexico (UNAM) served as external co-supervisor of this project and covered some of the fieldwork costs. RNA extraction and high-throughput sequencing was carried out in collaboration with Dr. Ricardo Saldana-Meyer at the New York University Langone Medical Centre who provided lab space. Mr. Tom Batstone from the University of Bristol and Dr. Zixia Huang from University College Dublin helped with initial bioinformatics analysis. Prof. Stephen Rossiter from Queen Mary University College London contributed to the idea of studying the genetics of migration and to the protocol design for the transcriptomic analysis. Dr. Maria Clara Arteaga from the Research and Education Centre of Baja California (CICESE) provided 119 cyt-b sequences and contributed to the interpretation of the results of the second chapter.

Angelica Menchaca Rodriguez

Signed [redacted] Date 2 April 2019

Dedication

Esta tesis va dedicada a la mujer que más admiro en este mundo: mi madre. Gracias tortuga blanca por enseñarme a seguir mis sueños, a escuchar a mi corazón y a ser valiente. Gracias por enseñarme a ser paciente y a entender que las alegrías más grandes de la vida llegan cuando uno menos las espera y a veces llegan simplemente en forma de pan. Gracias por enseñarme a trabajar duro por lo que más quiero, pero también a detenerme y admirar el mundo. Tu amor y tu bondad me hicieron ver el mundo con otros ojos, tu antes que nadie supiste que mi gran pasión eran los animales, y aunque convertí tu casa en zoológico nunca me quitaste la ilusión de tenerlos cerca. No hubiera llegado a este momento de mi vida si no fuera porque siempre has estado a mi lado apoyando mis grandes ideas. Gracias por ser más que una madre, has sido mi mejor amiga y mi alma gemela.

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Chapter 1. General Introduction

1.1 What is migration?

Migration is a type of movement that occurs across the animal kingdom in a wide variety of environments; it involves following a direction, is synchronised, demands preparation, and follows a cyclic pattern (Dingle and Drake, 2007). In some invertebrates, the migratory cycle may involve long distance movements over successive generations, as in the case of the monarch butterfly (*Danaus plexippus*) (Flockhart et al., 2013). Animals move between habitats to increase their fitness by exploiting favourable environmental conditions, to access high quality resources, and to avoid predators and disease (O'Connor et al., 2018; Dingle and Drake, 2007). Migration evolved independently in many lineages of birds with approximately 18.5% or 1,800 of all extant species being migratory (Rolland et al., 2014); in comparison, only about 3% or 40 species of all extant bats migrate distances greater than 50 km with only approximately 10 species moving over 1000 km in a single journey (Bisson et al., 2009; Fleming and Eby, 2003). Migration also occurs in other taxa such as fish, reptiles, amphibians, arthropods and marine invertebrates (Chapman et al., 2014). The phenomenon is continuously appearing and disappearing; animals often change their movement patterns and populations are known to transition from migration to residency and vice versa without necessarily showing an evolutionary pattern consistent with phylogeny (Alerstam et al., 2003).

Our knowledge of migration has resulted mainly from studies on birds made by both laymen and scientists; the first studies on bird migration date back to Aristotle (Berthold, 2001), and over the last 100 years we have accumulated vast information on migratory routes, timing and triggers (Gwinner, 1996; Vardanis et al., 2011), the influence that environmental change has on migratory behaviour (Newton, 2007; Berthold, 1999) and more recently the role that

genes are playing in migration (Liedvogel et al., 2011; Mueller et al., 2011; Steinmeyer et al., 2009). In contrast, our knowledge of migration in bats is relatively underrepresented compared with other taxa (Fleming and Eby, 2003).

Perhaps the elusiveness of bats, at least for the untrained person, their nocturnal habitats, and their lack of popularity has resulted in studies on bat migration lagging well behind to those of birds. Furthermore, the small size of bats and difficulty to track them have impeded gaining more knowledge about their migratory patterns and habits. Only very recently advances in GPS tracking technology have allowed researchers to gain insight on the movement ecology of flying foxes. For example, a study of Madagascan flying foxes (*Pteropus rufus*) followed the activity of this bat and confirmed its potential as a long-distance seed disperser that plays a role in the regeneration of threatened forests (Oleksy et al., 2015). Another study tracked Lyle's flying foxes (*Pteropus leylei*) and gained insight into the foraging behaviour of this bat in Thailand (Weber et al., 2015).

Recent advances in technology, including molecular methods such as genetic sequencing and technology for tracking animals, will improve the study of movement ecology at finer spatial-temporal scales (Bridge et al., 2011; Voigt et al., 2017). One of the most challenging matters of tracking animals with remote telemetry is the limiting size of devices because there are trade-offs among spatial resolution, battery life and the amount of additional weight that an animal can carry (Voigt et al., 2017). The most recent tracking units (1.1 g), available today, have allowed monitoring light levels, activity, temperature and movement patterns of >1000 km over 224 days of hoary bats (*Lasiurus cinereus*), which averages 13 to 14.5 cm long, has a 40 cm wingspan and a weight of 26 g (Weller et al., 2016). With these smaller tracking units

we could potentially track bats that weigh less than 30 g, such as the tequila bat *Leptonycteris yerbabuenae* studied in my thesis, and track its migratory movements over an entire season (Weller et al., 2016; Voigt et al., 2017).

1.2 Patterns of migration

Three general patterns of migratory behaviour in bats have been recognised: 1) *altitudinal migration*, which involves movements between areas that differ in elevation, often occurring over short spatial scales and involving few physiological adaptations (Mcguire and Boyle, 2013); 2) *regional migration*, where animals perform seasonal movements between hibernacula and maternity sites, without following a clear geographic trend but sometimes involving movements >500 km (Popa-Lisseanu et al., 2009; Krauel et al., 2018); and 3) *latitudinal migration*, where animals move during the spring following a clear migratory route and return to their breeding areas (Dingle and Drake, 2007). Furthermore, two migration systems describe the direction that New World fauna follow, with a Neartic-Neotropical system where animals breed in northern latitudes and overwinter in the warmer south; and a less studied Austral system where animals breed in the south and move north to overwinter, as is the case of many species of birds (Jahn et al., 2018; Dingle, 2008)

Furthermore, a broad spectrum of strategies ranging from residency, short-distance and long-distance movements can be identified within animal species (Alerstam et al., 2003). Migration can be *obligate* if individuals always migrate; however many migratory species are *partially migratory* where only a fraction of individuals migrate each year (Chapman et al., 2011). This provides a useful system for comparative studies on why individuals migrate (Shaw, 2016). *Differential* migration occurs when the migration patterns differ between age

and sex groups (Dingle and Drake, 2007). Although patterns and strategies can be very variable, migration is considered as a single phenomenon with four essential components: 1) a *migration arena* comprising the environment to which the organism is adapted (Dingle and Drake, 2007); 2) a *migratory syndrome* that includes all traits that enable the behaviour (Piersma et al., 2005); 3) a *genetic complex* that underlies the syndrome (Alerstam et al., 2003; Dingle and Drake, 2007); and 4) a *population trajectory* comprising the route followed, the timing of travel, and stopover points (Drake et al., 1995; Dingle and Drake, 2007).

1.3 Physiology of migration

To be able to move across environments, migratory animals need morphological and physiological adaptations that make travelling a cost-effective endeavour (Lennox et al., 2016). Flying organisms need aerodynamic wings that help to reduce the costs of locomotion, and they also need a modest body size to reduce the mass carried aloft (Piersma et al., 2005). Several morphological adaptations are observed in migratory bats; for example, they tend to have smaller brains than non-migratory bats (McGuire and Ratcliffe, 2011), they have reduced digestive organs, enlarged muscles and elevated fat storage to endure migratory journeys (McGuire et al., 2013). These morphological adaptations affect the physiology of these bats in a way that the high energy demands of migration are reduced (McGuire et al., 2013). Often, these changes are reversible within individuals, and hence involve phenotypic plasticity (Piersma, 1998).

Flapping is more energetically costly than running or swimming (Butler, 1991). To sustain flight, an animal needs to maintain a supply of energy to contract its muscles and needs to use this energy effectively until reaching a stopover or the final destination (Bradley et al.,

2017). Sustained periods of negative energy balance lead to decreases in total body and skeletal muscle mass and can cause nutrient sparing, where the body derives energy from fatty tissues (Carbone et al., 2012). Prolonged physical activity and elevated metabolic rate exacerbates inflammation and promotes the activation of immune cells leading to chemical modifications that stimulate a variety of cellular processes, including oxidative stress (Costantini et al., 2018; Skrip et al., 2015). Moving great distances and across complex landscapes is inherently challenging; failure to reach winter hibernacula or breeding sites quickly alters the composition of populations and can change the phenotypes of individuals by acting on particular parameters such as the direction they follow during migration and the time of the year when they do it; or may adjust many traits simultaneously, including diet, social structure and timing of reproduction (Dingle and Drake, 2007; Winkler et al., 2014).

Wing shape has been directly associated with foraging and migratory strategies in birds and bats; for example, studies on reed buntings (*Emberiza schoeniclus*) have shown that the wing shape of migratory birds differs substantially from sedentary individuals of the same species, with the migratory subspecies having larger, more convex wings than the sedentary one (Copete et al., 1999; Minias et al., 2015). Other studies on wing morphology have been useful to separate bat species based on their foraging and migratory strategies and show how performance, ecology and morphology are interrelated (see: Norberg & Rayner 1987; Bahlman et al. 2006; Marinello & Bernard 2014; Schmieder et al. 2015). Comparative analyses of bat species based on wing-shape differences have detected interspecific differences associated with foraging and flying strategies (see: Aldridge & Rautenbach, 1987; Marinello & Bernard, 2014; Rhodes, 1995). For example, wings that reach further toward the head might be beneficial to manoeuvre better in dense vegetation and may facilitate flight in

confined spaces (Schmieder et al., 2015). To my knowledge no studies have been able to find differences in wing shape related to migratory strategy, at the population level in bats.

1.4 The genetics of migration

Migration is an adaptive trait crucial for many species. However, the understanding of this phenomenon, particularly its genetic context, is somewhat limited (Krauel and Mccracken, 2013). Studies on the genetics of the migratory behaviour in vertebrates have been limited to understanding patterns in birds and in fish of economic importance (O'Malley et al., 2010; Hecht et al., 2012; Albert et al., 2006; Lundberg et al., 2013; Korsten et al., 2010; Jones et al., 2008). Interesting breakthroughs have been found in studies of the monarch butterfly (*Danaus plexippus*) (O'Malley et al. 2010; Zhan et al. 2011; Mueller et al. 2013). The migration of monarch butterflies appears to be under control of circadian clock genes and regulated at the epigenetic level so that different generations may exhibit different patterns of migration. Migration in mammals, however, has received less attention, probably because in contrast with the other taxa, most species are terrestrial and only a small fraction of them undertake this event (Fleming and Eby, 2003). To my knowledge, no studies have addressed the question of which genes, metabolic pathways and cellular processes are controlling the physiological mechanisms of migration in mammals.

The underlying mechanisms involving molecular, physiological or endocrinological adaptations for migration remain mostly unknown (Mueller et al., 2011). Studies of the genetic architecture of migration would provide meaningful biological indicators of a species' potential to survive in the face of environmental change, and could potentially serve as a robust framework to make educated decisions in conservation and management (Allendorf

et al., 2010). This knowledge would also provide insight into how to better evaluate and mitigate the impacts that climate change will have on populations.

1.5 Triggers of migration

Migration is triggered by a complex combination of internal and external cues (Shaw, 2016). An individual's body condition (e.g. having high levels of internal energy reserves) may also influence the timing of migration of certain species toward their breeding grounds such as in salmon (*Oncorhynchus spp*.) (Brodersen et al., 2015). The changes in day length, or photoperiods, is commonly used as a cue for bird migration in temperate regions (Wingfield, 2005), and the length of the migratory route appears to be encoded by the circannual clock of the bird (Liedvogel et al., 2011). Furthermore, environmental temperature, and consequently climate change, also plays a role as determinant for migration in fish (Kuczynski et al., 2017), insects (Chapman et al., 2015) and amphibians (Dervo et al., 2016). If environmental cues driving migration are linked to climate variables, such cues could aid in predicting whether and how changing climatic conditions will impact upon migration (Shaw, 2016). Predicting whether genetic change and phenotypic plasticity can match the accelerated environmental change will depend on improving our understanding of how genetic and phenotypic pathways for migratory flexibility work and interact (Winkler et al., 2014).

1.6 Evolution of Phyllostomidae

Leaf nosed-bats (Phyllostomidae) are part of one of the most diverse families of bats that inhabit Central and South America. Leaf-nosed bats have undergone adaptive radiation that has been considered unparalleled among other mammals in terms of ecological and

morphological diversity (Montero and Nogueira, 2011). An insectivorous ancestor from the Eocene gave rise to the 53 genera of Phyllostomids found today (Simmons, 2005). The family diversified in species with six distinct feeding strategies: sanguivory, insectivory, frugivory, nectivory, carnivory (feeding on vertebrates) and omnivory (Baker et. al., 2010) (Table 1, Figure 1.1) This ecological diversity and specialisation is the product of resource partitioning and is responsible for the high local species richness (over 160 spp.) (Monteiro and Nogueira, 2011). The evolution of specialised diets created and ecomorphological diversification associated to functional demands, which determined changes in the shape of the cranium, mandibular and probably wings.

Within Phyllostomidae, the Subfamily Glossophaginae is comprised by primarily nectar feeding bats (some may also feed on fruits or insects depending). Members of these subfamily diverged Glossophagines diverged from other phyllostomid bats 21.55 (23.4–19.7) mya, with the last common ancestor of all 12 genera being present 17.48 (20.8–14.2) mya (Baker et al., 2019). This results in a maximal estimate of nine million years to evolve from a primarily insectivorous omnivore into a nectar feeder (Baker et al., 2019) Table 1, Figure 1.1.

Table 1 Characteristics of nine clades of phyllostomid bats that are hypothesized to have evolved from a common ancestor. Intraclade age is the time that the clade separated from all other phyllostomids. Change from common ancestor is based on an overview of general knowledge. Modified from Baker et al., 2019.

Food Source	Age (mya)	Intraclade age (mya)	Time in common ancestor	No. of genera	No. of species
insects / plants	29.5	na	na	1	2
insects / plants	26.7	23.2	3.5	2	8?
blood	25.9	21.7	4.2	3	3
insects / fruit	23.3	na	na	1	6
omnivore /vertebrate / insects	22.3	21.1	1.2	9	20
nectar / pollen/ fruit / insects	21.4	17.4	4	13	31
nectar /pollen / fruit/ insects	18.5	11	7.5	4	10
Insects / fruit	18.5	17.2	1.3	3	13
fruit	18.5	16.2	2.3	19	68

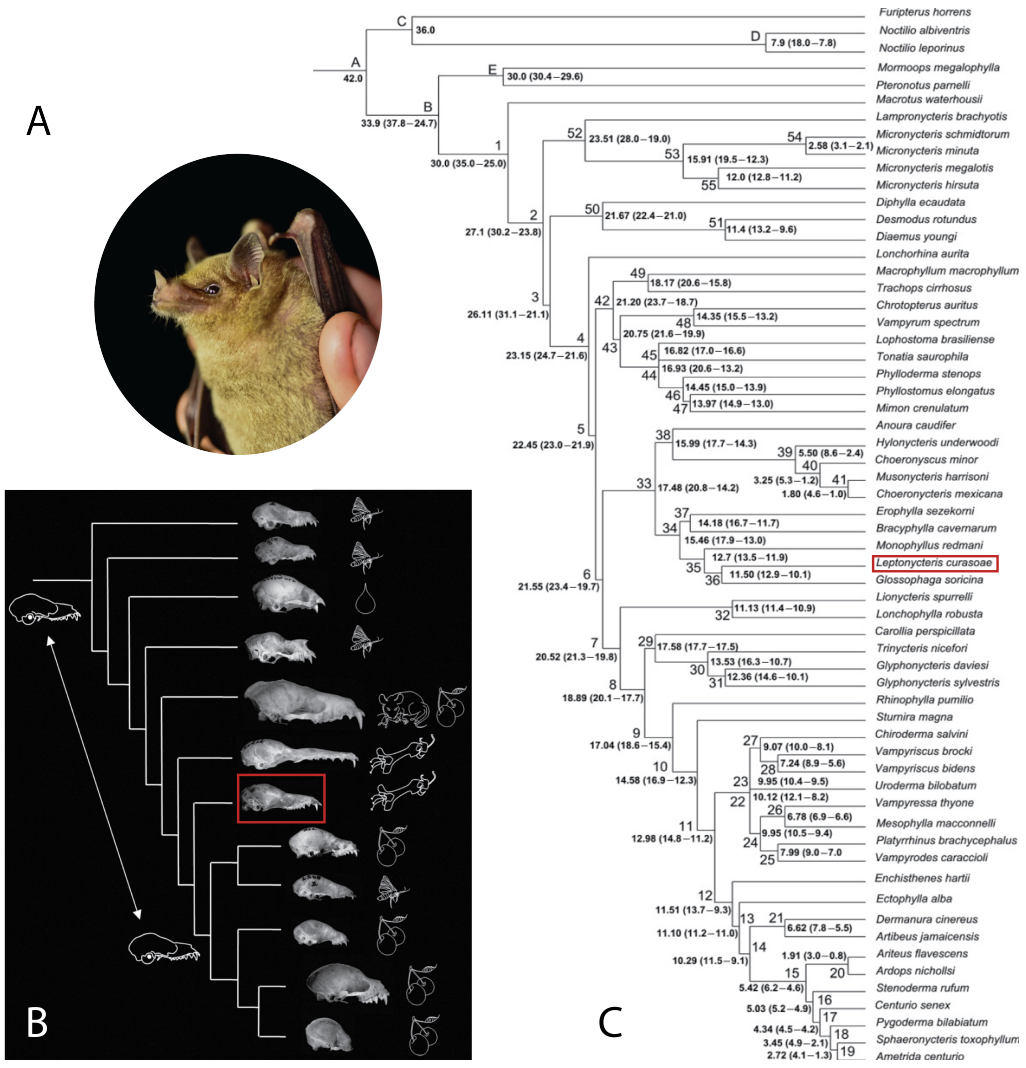


Figure 1.1 Evolution of the Family Phyllostomidae. A) profile picture of a *L. yerbabuenae*, B) a phylogenetic perspective of cranial variation in the family with primary food source, C) Estimates of geological age mapped on phylogenetic tree of mitochondrial and nuclear genes after Baker et al., 2003. Modified from Baker et al., 2019.

1.6.1 Natural History of the tequila bat (*Leptonycteris yerbabuenae*)

The lesser-long nosed bat, more widely known recently as the tequila bat (*Leptonycteris yerbabuenae*; Figure 1.2) is a medium-sized phyllostomid of about 26 grams that feeds on nectar, pollen and fruits (Cole and Wilson, 2006). It ranges from southern Arizona and New Mexico in the United States to Honduras and El Salvador (Cole and Wilson, 2006). The species

is considered “Near Threatened” in the IUCN Red List of Endangered Species (IUCN, 2018)(**Error! Reference source not found.**).

Feeding habits have determined the migratory behaviour of the species, as it moves every year in search of favourable ecological conditions and resources during the reproductive period (Wilkinson and Fleming, 1996). The ecology and population dynamics of *L. yerbabuenae* have shaped its co-evolution with several plant species (Fleming and Nassar, 2002). *L. yerbabuenae* is a primary pollinator of columnar cacti (*Stenocereus spp*.) and paniculate agaves (*Agave spp.*), including the blue agave (*Agave tequilana*) used to make tequila and mescal, and other plants that represent distinctive elements of the flora of northern Mexico and the southern U.S. (Peñalba et al., 2006; Rocha et al., 2006). *L. yerbabuenae* has been considered as a ‘keystone mutualist’ and a ‘mobile link’ between habitats (Horner et al., 1998; Peñalba et al., 2006; Ober and Steidl, 2004; Fleming et al., 1993; Fleming, 1994). Moreover, the species has recently gained popularity due to the implementation of a ‘bat-friendly' certification awarded to tequila and mezcal producers who adhere to norms established by the Tequila Interchange Project to maintain bat-mediated gene flow among populations of blue agave (*A. tequilana*) (Medellin, R. 2014 pers. comm.).

L. yerbabuenae roosts in caves and mines, often in colonies numbering several thousand individuals, and its population comprises a large number of subpopulations ranging from central California, southern Arizona, and New Mexico to Honduras and El Salvador (Cole and Wilson, 2006) (**Error! Reference source not found.**). *L. yerbabuenae* is considered a long-istance migratory species as it performs seasonal movements of >1700 km in each direction. It is also considered a *partially* migratory species as its populations divide into a migratory and a resident group (Morales-Garza et al., 2007; Cole and Wilson, 2006; Rojaz-Martinez et

al., 1999; Wilkinson and Fleming, 1996) During spring and summer, pregnant females move from the centre of Mexico along the Pacific Coast to north-western Mexico and south-eastern Arizona (**Error! Reference source not found.**). The blooming of over 30 species of columnar acti, agave, and yucca plants create nectar corridors that provide the bats with food during their migration, and in turn, the plants are pollinated (Heithaus, 1982; Horner et al., 1998). Bats arrive at warm caves in the desert and establish maternity colonies where they stay until the end of the summer, then return south following blooming agave corridors along the Sierra Madre Occidental (Ramirez, 2011) (**Error! Reference source not found.**). Migratory bats form he largest maternity colonies known for the species, and interestingly they are the only group of females that aggregates their young in "patches" of hundreds of newborns to maintain adequate developmental temperatures and perhaps to safeguard the pups from predators (Iñarritu-Castro, 2017) (Figure 1.6), while the non-migratory females found in Southern Mexico do not aggregate the pups in patches but rather leave them perching on their own (pers. obs. 2015-2017).

The natural history of male *L. yerbabuenae* has been less studied; they are believed to disperse across the centre of Mexico and mate with individuals during the autumn and winter (Nassar et al., 2008; Rojaz-Martinez et al., 1999; Stoner et al., 2003) (**Error! Reference source ot found.**). The males produce sebaceous patches during spermatogenesis and the mating period, suggesting involvement of olfaction in communication and sexual selection (Nassar et al., 2008). A study on seasonal colonies of migratory *L. yerbabuenae* in the Baja California peninsula examined the genetic structure and proposed that current patterns of genetic differentiation are caused by matrilineal gene flow with only subtle signatures of population structure mediated by males (Arteaga et al., 2018)(**Error! Reference source not found.**). The

roup found in southern Mexico also forms large maternity colonies but does not undergo latitudinal migration, instead they perform altitudinal movements between areas that differ in elevation depending on resource availability (Herrera-Montalvo, 1997; Morales-Garza et al., 2007), and thus can be classified as a resident or altitudinal migrant population that does not move across long-distances as the northern population does (**Error! Reference source not ound.**).

Thesis overview

1.7 Aims and objectives

My thesis aims to 1) generate evidence regarding the genetic basis of migration in bats and 2) fill some of the knowledge gaps in migratory research. The specific goals of this project are to:

- obtain a high-quality *de novo* transcriptome of blood and brain tissues of *L. yerbabuenae* for the first time and with it lay the foundation for future research on its evolution, especially as the genome is as yet unavailable for this bat.
- apply a non-lethal method to study the evolution, immunology and ecology of a non-model organism using whole blood transcripts.
- answer key questions about the genetic basis of migration in *L. yerbabuenae* and provide molecular information on the underlying mechanisms that confer bats the ability to migrate.
- compare migratory and resident populations from morphological and genetic perspectives to characterise the main phenotypic differences and to identify migratory candidate genes.

- determine whether phenotypic differences in the wing shape of migratory and non-migratory individuals of *L. yerbabuenae* occur, and if so to correlate genetic data with morphological variation.
- contribute information that could be useful to understand if environmental variation and potential future change will alter the behaviour of the species and provide insights on adaptive capabilities in a changing environment.
- apply genomic knowledge to improve conservation and management strategies in a species of bat by defining relevant conservation units. And to open the door for future research on the genomics of migration.
- investigate mechanisms facilitating adaptation and provide information to maintain better genetic connectivity between populations that could translate into long-term conservation actions such as management of resources and protection of critical areas.

1.8 Thesis outline

This thesis includes three data chapters followed by a general discussion of the broader evolutionary implications of migration in *L. yerbabuenae* as a result of genetic and phenotypic variability.

In chapter 2, I use mitochondrial markers to investigate genetic structure in three populations of *L. yerbabuenae,* including one migratory population composed of females, one non-migratory population consisting of males and one non-migratory population consisting of females. Each sampled population occurs along the range of the species in Mexico and thus the sampling broadly covered the entire latitudinal range of the species in Mexico. In this

chapter, I test the hypothesis that matrilineal gene flow is maintained between populations and results in low population structure and high levels of gene flow; I use this information to corroborate that the three populations belong to the same species and to infer patterns of historical and recent matrilineal gene flow using two mitochondrial markers.

In chapter 3, I maintain a focus on characterising the migratory and the non-migratory populations of *L. yerbabuenae* and use traditional morphometrics and geometric morphometrics to investigate sexual dimorphism and variation of wing shape explained by the migratory behaviour and foraging strategies of each group. In this chapter, I test the hypothesis that wing shape is adaptive and predict that migratory bats have wings better suited for long-distance flight such as narrow wings as a result of higher aspect ratio for flight endurance and higher wing loading for fast flight than the non-migratory bats.

In chapter 4, I continue researching the differences between the migratory and non-migratory populations and use transcriptomics to compare the profiles of blood and brain transcriptomes of females of *L. yerbabuenae*. In this chapter, I test the hypothesis that gene expression differences are correlated to the migratory behaviour and that genes previously associated with migration in other taxa, such as circadian clock genes, will be up-regulated in the migratory population and related to the entrainment to the annual cycle by changes in photoperiod. With these data, I propose a list of candidate genes associated with migration. I discuss the potential link of circadian clock genes to migratory behaviour and provide insight on other likely genes responsible for the onset and evolution of migration.

Figure 1.2 The tequila bat (*Leptonycteris yerbabuenae*) profile and pollinating a saguaro plant

Figure 1.3 The Pinacate peaks and Gran desierto de Altar Biosphere Reserve in the Sonoran Desert, Mexico. Photo credit: A. Menchaca

Figure 1.4 View from the inside of Los Laguitos cave in Chiapas, Mexico. Remains of low land forest are observed and vandalism inside the cave. Photo credit: A. Menchaca

Figure 1.5 Female bats returning to El Pinacate cave. Pregnant females can be observed.

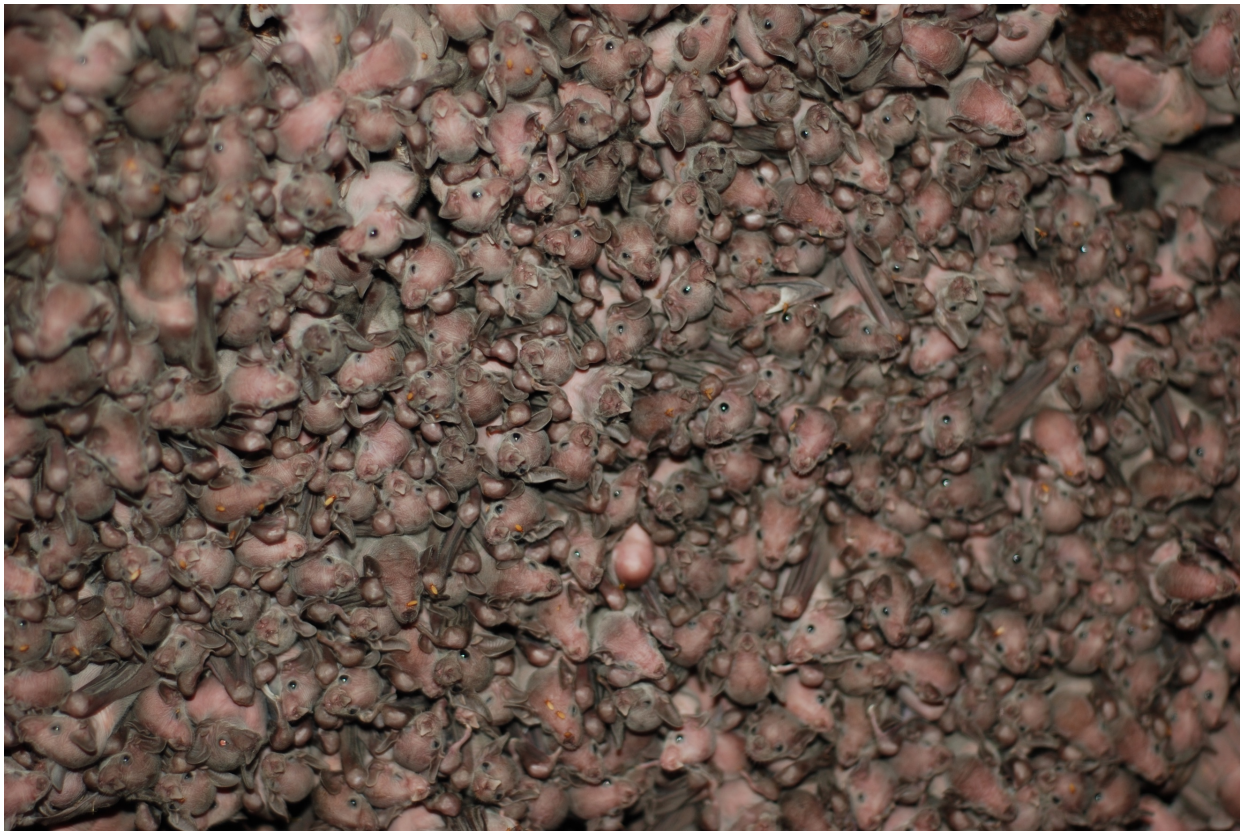


Figure 1.6 Newborns aggregation of hundreds of individuals. Photo credit: A. Menchaca

Chapter 2. Mitochondrial haplotype diversity in the tequila bat (*Leptonycteris yerbabuenae*)

Abstract

The tequila bat (*Leptonycteris yerbabuenae*) has been the focus of intense research over the last 20 years. Its close relationship to economically important plants used in the production of tequila and mezcal has raised awareness of its importance as a pollinator and migratory species. However, most studies have been centred on the northern migratory populations, and little is known on the most southern non-migratory ones. Here, I used partial sequences of mtDNA cytochrome b and Control Region to infer patterns of differentiation among four populations across its distribution. I test the hypothesis that a single panmictic population exists as a result of long-distance migration and only subtle patterns of population structure occurs given geographical constrains and differences in breeding timing. My analysis suggests: 1) panmixia exists among northern, central and southern populations; and 2) weak genetic differences between northern and southern populations occurs with a trend for differentiation, 3) one single species is recognised but two Evolutionary Significant Units (ESUs) are proposed based on genetic, migratory behaviour and ecological distinctions.

2.1 Introduction

2.1.1 Species concepts and conservation units

One of the main challenges in conservation biology is to identify potential conservation units (CUs) or evolutionarily significant units (ESUs) (Smith and Wayne, 1997). Such units correspond to populations with recently shared histories that should be managed separately to preserve their biological diversity (Smith and Wayne, 1997; Funk et al., 2012). Usually, management programs are based on taxonomic classifications which were based on a small number of specimens and solely on morphological traits (Funk et al., 2012). Moreover, in many cases, the taxonomy at the subspecific or specific levels needs to be revised and can be improved with molecular tools (Frankham, 2010). The evolution of closely related taxa, as potential ESUs, has been linked to questions of speciation; therefore, what constitutes an ESU is inherently defined by the concept of "species" being used (Vogler and Desalle, 1994).

Under the "biological species concept", as defined by Mayr in 1942, species are recognised as groups of organisms with the potential to interbreed and which are isolated from other groups (Mayr, 1942). Conservation units have been defined under this concept as reproductive isolation is an objective and unambiguous criterion. However, establishing the reproductive compatibility of related subgroups is extremely difficult, and consequently, the relative degree of morphological and genetic differentiation is taken as a proxy to define populations. To complicate matters further, taxonomists and conservationists have not agreed on the levels of genetic variation that separate groups of organisms and would unequivocally classify them as different species or conservation units (Vogler and Desalle, 1994).

Under the more recent “phylogenetic species concept”, proposed by Cracraft in 1982, a species is defined as the smallest set of organisms sharing an evolutionary history that can be distinguished from each other by phenotypic variation (Cracraft, 1983); more recently this definition has been expanded to recognise species not only on phenotypic differences but also genotypic, behavioural and ecological variation (Vogler and Desalle, 1994; Palsbøll et al., 2007; Crandall et al., 2000). Again, defining what constitutes enough distinction can be problematic, but under this concept, it is easier to define ESUs. If shared characters in all members of a proposed population are not detectable in another, such populations may merit separate protection (Vogler and Desalle, 1994). As the impact of using the phylogenetic species concept can lead to an increase of the number of species and complicate the identification of taxonomic distinctiveness even further, in this study, I will use the biological species concept to define what constitutes a species but will apply the phylogenetic concept of species and consider aspects of the behaviour, natural history, sex and habitat requirements to establish ESUs for conservation purposes.

2.1.2 The use of mitochondrial DNA (mtDNA) in conservation

The analysis of mtDNA polymorphisms represents the most commonly used means for revealing phylogenetic relationships among closely related species and populations (Boore et al., 2005; Ivanova et al., 2012) The mitochondrial genome comprises a circular, double-stranded DNA molecule of about 20,000 base pairs (bp) containing 2 rRNA genes, 13 protein-coding genes, 22 tRNA genes, and a non-coding region of ~1,000 bp known as Control Region (CR) or D-loop (Smith and Wayne, 1997). As the mtDNA is transferred without recombination through maternal lineages, it is considered haploid (Alberts et al., 2002). It is a very well-

known molecule for which a very large database is available for an increasing number of organisms (Ivanova et al., 2012).

Relying solely on mtDNA is not sufficient to differentiate cryptic species, or to understand the complete phylogenetic and evolutionary history of species. However, the analysis of highly polymorphic genetic regions of mtDNA combined with analysis of diploid markers such as microsatellite nuclear sequences has allowed the recognition of bat species with a very recent evolutionary divergence that lack noticeable morphological distinctive characters (see: Clare et al. 2013; Rakotoarivelo et al. 2015; Tu et al. 2017). At the intraspecific level, mtDNA alone has been useful to recognise conservation units in barbastelles (*Barbastella barbastellus*) (Rebelo et al., 2012). Combining mtDNA and nuclear DNA has been useful to recognise conservation units in greater horseshoe bats (*Rhinolophus ferrumequinum*), long-fingered bats (*Myotis capaccinii*) and bent-winged bats (*Miniopterus schreibersii*) (Bilgin, 2012).

Furthermore, distinct conservation units have even been defined based on sex-specific habitat requirements and behaviour. For example, in the parti-coloured bat (*Vespertilio murinus*) where females rely more on lakes while foraging and males display more flexibility in habitat use (Safi et al., 2007). This niche segregation of the two sexes has been found to have a profound impact on the extinction risk of the species as a whole. Thus, the differences in habitat requirements are useful to define two distinct conservation based on sex.

2.1.3 Defining populations of *Leptonycteris yerbabuenae*

Being a migratory and widely distributed species, defining the boundaries of the populations of *L. yerbabuenae* has been somewhat problematic. Several analyses on population genetics

based on mitochondrial DNA (mtDNA), random amplified polymorphic DNA (RADP), and microsatellite loci have helped elucidate if the species forms a single panmictic population across its range or if there is evidence of population structuring within and between colonies. Furthermore, these studies have also shed light on patterns of contemporary migration and evolution of the species (Wilkinson and Fleming, 1996; Morales-Garza et al., 2007; Ramirez, 2011; Arteaga et al., 2018).

Most of the information about the species arises from studies of the huge maternity colonies that it forms. Females of *L. yerbabuenae* are monoestrous and give birth to a single pup each season (Cole and Wilson, 2006). According to their reproductive season, two populations of females have been recognised: one population that gives birth during spring in northern Mexico and southern Arizona deserts, and a second one that gives birth during winter in south-central Mexico (Wilkinson and Fleming, 1996; Fleming and Nassar, 2002; Ceballos et al., 1997). The first population is a long-distance migrant and conducts a journey spanning roughly 1,700 km following a nectar corridor from central Mexico to suitable roosting maternity caves in the desert to then return in the early autumn (Figure 2.1) (Wilkinson and Fleming, 1996; Cole and Wilson, 2006). The second population is considered resident and remains at latitudes lower than 21° N and moves altitudinally depending on food availability (Figure 2.1) (Valiente-Banuet et al. 1996; Rojaz-Martinez et al. 1999; Morales-Garza et al. 2007). Evidence of such altitudinal movements was obtained with capture-recapture methods (Rojas-Martinez, 2001; Galindo et al., 2004), and carbon stable isotope analyses (Herrera-Montalvo, 1997).

Many aspects of the population ecology and whereabouts of males throughout the year remain poorly understood. Reports have proposed a transitory population with great abundance from October to January in the Centre of Mexico in Chamela, Jalisco (Stoner et al., 2003) and in Colima in central-western Mexico during June (pers. obs. 2017), and a second one in the Tehuacan Valley in central Mexico during August (Herrera-Montalvo, 1997) (Figure 2.1). These populations are geographically intermediate between the northern migratory and the southern non-migratory female populations (Figure 2.1).

Observations of males with enlarged testes have also been reported in the north in Sonora during June (Calva O. 2018, pers. comm.). More recently, males have also been found in the Baja California Peninsula during the summer (Arteaga et al., 2018). Reports of males using hummingbird feeders have been made yearlong near Tucson and could have an effect associated with urban development (US Fish and Wildlife Service, 2016). This permanent availability of resources could impact their migratory/dispersing behaviour by discouraging them to move to breeding sites during the autumn and winter.

Male-mediated gene flow has been suggested between the northern and southern populations and two reproductive seasons have been proposed (Stoner et al., 2003; Rojaz-Martinez et al., 1999). A study on the male reproductive cycle of *L. yerbabuenae*, based on histological and external characteristics, demonstrates males have a single peak spermatogenesis period and no possibility of long-term sperm storage, with a few individuals maturing earlier or later than the rest of the population (Rincón-Vargas et al., 2013). The study rejects the hypothesis that the same male has the potential of reproducing twice in a single year with a female from each population, but suggests the hypothesis of parturition

asynchrony between populations due to delayed implantation or arrested gestation (Rincón-Vargas et al., 2013). Furthermore, this study recorded the appearance of a dorsal patch that develops in males and that has unique chemical characteristics possibly involved in female attraction during the summer months (June-July) indicating that the colony of males located in east-central Mexico (Figure 2.1) mate with the females during this time of the year. Evidence of breeding and active males (with enlarged testes) have been found in west-central Mexico from Oct-December (Ceballos et al., 1997; Stoner et al., 2003) (Figure 2.1). Interestingly, Stoner et al. (2003) also observed males with enlarged testes in September–January and May and June (which coincides to the time when males in east-central Mexico are mating), suggesting that males could be moving across Mexico in search of reproductively active females (Figure 2.1).

A study on the mitochondrial Control Region (CR) from bats at 13 locations across the range of *L. yerbabuenae* revealed two clades with weak population structure, corresponding to migratory individuals from the north regions of Mexico and non-migratory individuals from south-central Mexico, and suggesting that movements between sites has maintained considerable haplotype diversity in the species (Wilkinson and Fleming, 1996). The authors, also proposed two migratory corridors: one along the Pacific coast and a second one inland along the Sierra Madre Occidental (western mountain range). Furthermore, movements along the Sierra Madre Oriental (eastern mountain range) by southern and central bats has been suggested (Wilkinson and Fleming, 1996; Morales-Garza et al., 2007; Rojaz-Martinez et al., 1999) (Figure 2.1) but not demonstrated unequivocally e.g. by using radio tracking. A study based on 12 microsatellite loci and sequences from the mitochondrial Control Region (CR) of northern and central populations recorded high levels of genetic diversity and gene flow;

suggesting a single panmictic population throughout Arizona (southwestern US), Sonora (north-western Mexico) and Jalisco (on the Pacific coast) (Figure 2.1). Subtle genetic structure was also observed, which could indicate the existence of a second population in central and southern Mexico (Ramirez, 2011).

A third study examined the relatedness of six populations (three sites corresponding to south-central Mexico and three from north-western Mexico) of *L. yerbabuenae* using random amplified polymorphic DNA (RAPD) across the species range and confirmed the presence of two well-defined populations: one along the Pacific coast ranging from northern Mexico, including roosts found in Baja California and Sonora (northern Mexico), Jalisco and Colima (central Mexico), and a second one in south-central Mexico including roosts found in Oaxaca, Chiapas, Tehuacan Valley, Puebla, Morelos and Hidalgo (Morales-Garza et al., 2007)(Figure 2.1). Furthermore, the authors propose the existence of a third population in the southwestern United States and north-western Mexico, which has recently been studied using microsatellite and mtDNA data (Arteaga et al., 2018).

In conjunction, these genetic-based studies demonstrate a separation between the migratory individuals found in northern Mexico and the southern United States versus the non-migratory individuals found in south-central Mexico. The search for better quality resources and environmental conditions is one of the most critical factors that has shaped the migratory patterns of *L. yerbabuenae* and which is directly influenced by the flowering cycles of columnar cacti and paniculate agaves in the north and by the year-round availability of resources in south-central Mexico. The differential resource availability in different parts of the range of *L. yerbabuenae* has divided the species into a long-distance migratory population

and a resident population, which have undergone specific selection contributing to the development of particular migratory behaviours, and ecological and reproductive characteristics.

In this thesis, I define three populations that are arbitrarily defined based on their geographical location at particular times of the year as so: 1) a northern population named "Pinacate", represented by migratory females occurring in Sonora and Baja California, 2) a central population named "Colima", represented by non-migratory males occurring in Colima; and a southern population named "Chiapas" represented by non-migratory females occurring in Chiapas.

2.2 Objectives

The objectives of my study include the following: 1) confirm the species' taxonomy as a single species by using genetic markers; 2) determine population structure and infer matrilineal convergence of *L. yerbabuenae* between colonies in northern, central and southern Mexico using mtDNA; 3) determine levels of genetic similarity and differentiation between and among these three populations; 4) estimate contemporary migration rates; and finally; 5) provide molecular evidence to define ESUs to aid in the conservation of *L. yerbabuenae*.

2.3 Hypothesis

Due to geographical constraints between southern Mexico and central Mexico, I hypothesise that the individuals from the north will be genetically more similar to the individuals from the centre and less similar to the individuals from the south. I predict that F_{ST} estimators (a

measure of population differentiation due to genetic structure) will be low to moderate for northern groups, as is typical for migratory species, and will be higher for the males in central Mexico and the females in southern Mexico, which are non-migratory. I predict very low patterns of population structure due to gene flow maintained by males but high levels of population differentiation due to isolation by distance, particularly between northern and southern populations.

2.4 Materials and methods

2.4.1 Study sites and sample collection

I collected 3mm wing punch samples of *L. yerbabuenae* corresponding to three study sites (n=20 for each site; Figure 2.1): the first site is located in the Sonoran Desert in northern Mexico (A), the second one in Colima (C) in mid-western Mexico and the third one in Chiapas (D) in southern Mexico. An additional site was included in the analysis which corresponds to sequences generated from migratory bats from Baja California (Figure 2.1) and were provided by Arteaga (2018). The roost in Sonora corresponds to a maternity roost cave of female migratory bats, the one in Chiapas corresponds to a female maternity roost cave of non-migratory bats, and the one in Colima to a roost cave of non-migratory males (Figure 2.1).

Bats were captured at the entrance of their cave roost using mist nets of variable lengths on movable poles. We controlled the number of captures by opening or closing the nets depending on the activity of bats during particular times of the night and always after they had returned from feeding (between 02:00-05:00 hrs). Individual bats were held inside bags until they were processed and released unharmed right after data collection. Wing skin

samples were collected with 3mm biopsy punches and stored in RNA-later buffer (Thermo Fisher Scientific, Baltics UAB) at room temperature until they were transported to the lab and kept at -20°C until DNA extraction.

The necessary collection permits were obtained in accordance with The Mexican Secretariat of Environment and Natural Resources (SEMARNAT) for populations at risk or in critical habitat, permit number SGPA/DGVS/03946/15. And imported in accordance with 42 CFR Section 71.54 of the Public Health Service Foreign Quarantine Regulations issued by the Department of Health and Human Services, Centre for Disease Control and Prevention of the United States of America, permit number 2017-04-136.

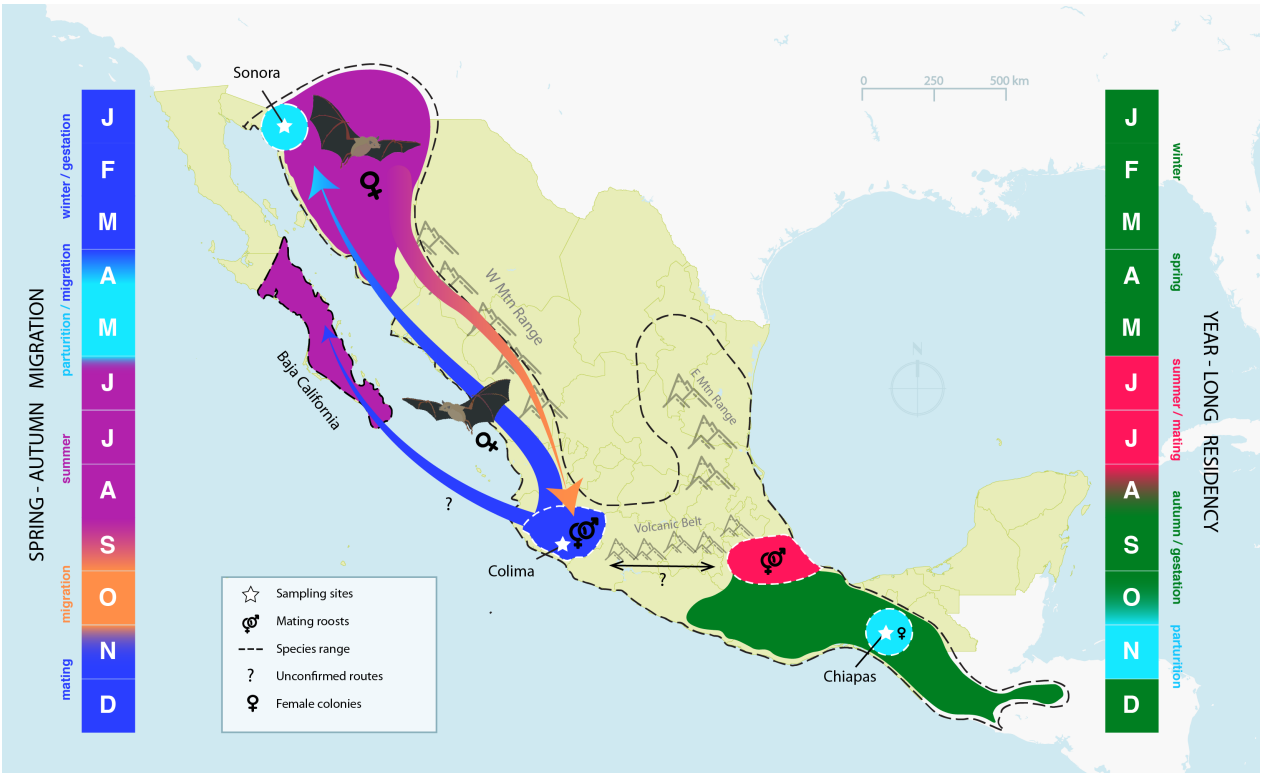


Figure 2.1 Map of migratory routes, distribution and reproductive seasons of *L. yerbabuenae.* Timelines of activities during a year of the migratory population (left) and the resident population (right). Female and mating roosts are depicted with sex symbols. Unconfirmed migratory and dispersion routes are shown as arrows with a question mark. Documented spring and autumn migratory routes are shown with thick arrows. Stars represent sampling sites in northern (Sonora), central (Colima) and southern (Chiapas) Mexico. Black dashed line represents the species distribution according to IUCN-2018.

2.4.2 DNA extraction and amplification

DNA extracted from wing biopsy punches was used to amplify a fragment of cytochrome b (cyt-b) and Control Region (CR); these two mitochondrial regions have been previously used in studies on the molecular systematics and population genetics of *L. yerbabuenae* (Arteaga et al., 2018; Wilkinson and Fleming, 1996; Ramirez, 2011).

I extracted total genomic DNA using the DNeasy Blood & Tissue extraction kit (Qiagen, GmbH - Germany), following manufacturer's recommendations with additional optimisation steps. I checked the quantity of DNA in every sample utilizing a Qubit 3.0® Fluorometer (Thermo Fisher Scientific). For samples that yielded less than 3ng/ul of DNA, I performed a Whole Genome Amplification (WGA) using the Illustra Ready-To-Go GenomiPhi V3 DNA Amplification Kit (GE Life Sciences, Freiburg, Germany) following manufacturer recommendations. WGA technology allows copying nanogram quantities of the original DNA template with high accuracy and provides high yields of whole genomic DNA that can be used in downstream applications (Czyz et al., 2015). After WGA, I measured the concentration of DNA again with the Qubit 3.0® Fluorometer. Samples were stored at -20°C until PCRs were performed.

Although, WGA is not necessary for mtDNA amplification (enough genomic DNA can be obtained with traditional extractions methods), I included this step to overcome the problem of limiting amounts of available DNA. I tested the reliability of WGA in downstream analysis by comparing cyt-b sequences obtained from five samples treated with WGA and without it. The sequencing data was identical in both cases thus concluding that WGA-treated samples resulted in high-quality DNA in sufficient quantities for successful sequencing.

For cyt-b analysis, I sequenced a region of ~350 bp with primers that have been used for species identification in a variety of mammal species: L14841 5'-AAAAAGCTTCCATCCAACATCTCAGCATGATGAAA-3' and H15149 5'-AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA-3' (Kocher et al., 1989). I obtained 119 additional complete cyt-b sequences from 5 sampling sites in Baja California (Figure 2.1) from Arteaga (2018) and compared them with the ones generated in this study. For CR analysis, I sequenced a region of ~550 bp with primers DLH16750 5'-CCTGAAGTAGGAACCAGATG-3'and THRL16272 5'-CCCGGTCTTGTAAACC-3' (Li et al., 2006).

Each polymerase chain reaction (PCR) was performed with the Qiagen HotStartTaq Plus Master Mix (Qiagen, GmbH Germany) in a 20 ul final volume reaction containing 1 unit of Taq, 1x PCR buffer, 200 uM dNTP, 1.5 mM MgCl2, 0.25 uM each primer, and 25-250 ng of DNA. Thermocycling conditions included an initial denaturation at 95°C for 5 min, then 35 cycles with denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, elongation 72 °C for 30 s and a final elongation of 72 °C for 10 min. All amplifications were performed in an Eppendorf Mastercycler nexus Thermal Cycler. PCR products were resolved in 3% agarose gels. Successful amplifications were cleaned with ExoSAP-IT (Life Technologies Europe BV), and Sanger sequenced in both directions at the School of Biological and Biomedical Sciences at Durham University.

2.4.3 Species Identification and haplotypes diversity patterns

I aligned and edited all sequences using Geneious v11.0.5 (Kearse et al., 2012) (Figure 2.2); apparent heterozygosities were corrected according to the IUPAC nucleotide ambiguity code. I then selected a consensus region containing 95% or more variation within the samples. I did

a BLAST search for all consensus sequences to confirm the species identity. I conducted statistical analysis of genetic variation, haplotype inference (h), plotted statistical parsimony networks via the median-joining method, estimated nucleotide diversity (π), haplotype diversity (Hd), polymorphic sites (S), and genetic differentiation (Φ_{ST}) with PopART v1.7 (Leigh and Bryant, 2015). I used the Φ_{ST} estimated in PopART to calculate pairwise genetic differences among *L. yerbabuenae* by grouping Pinacate and Baja California as the northern group and analysed bats from these sites separately from Colima (central group) and Chiapas (southern group) (Figure 2.1).

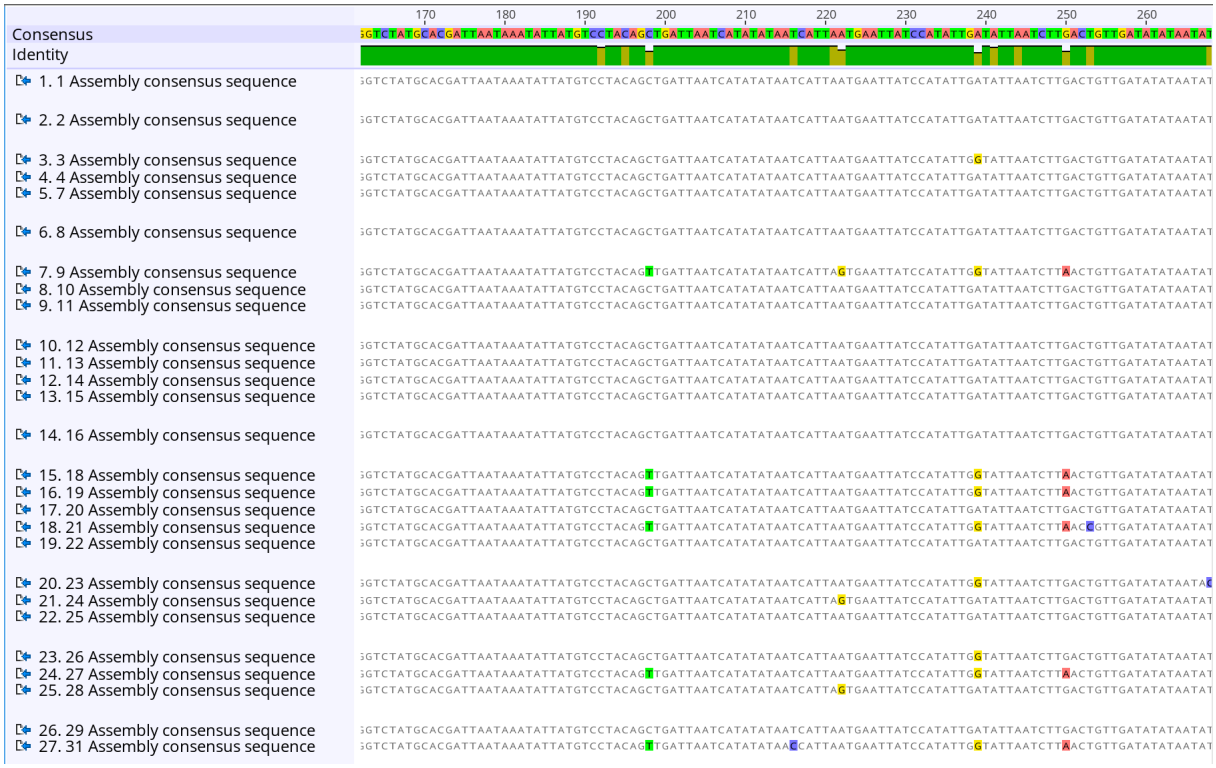


Figure 2.2 Global sequence alignment using Geneious software at 65% similarity. Nucleotide disagreements are highlighted in red, green, yellow or blue.

2.4.4 Test of neutrality and estimates of population expansion

I explored the demographic history of *L. yerbabuenae* by estimating Fu's neutrality test (Fu, 1997) and Tajima's *D* test (Tajima, 1989) with 10,000 permutations, and performed an analysis of mismatch distribution based on before and after population expansion (θ_0 and θ_1) and the time since population expansion (τ) expressed as units of mutational time with DnaSP v5.1 (Rozas et al., 2017).

I estimated the species divergence time using cyt-b with a Bayesian MCMC approach as implemented in BEAST v2.5.1 (Drummond and Rambaut, 2007). The program ran with a relaxed lognormal clock and the Yule process as the tree prior with 10,000,000 generations (Drummond et al., 2006). I applied a TN+F+I substitution model as predicted by IQ-TREE v1.6.7 (Nguyen et al., 2015). The fossil clock calibration was done using the TN93 model with 4.7% per million years as calculated for *Myotis* spp. (Ruedi and Mayer, 2001) because the generation time for *L. yerbabuenae* (or other of the same genus) has not been estimated yet.

2.4.5 Genetic differentiation

I explored hierarchical partitioning of genetic variation with an Analysis of Molecular Variance (AMOVA) and calculated estimates of gene flow (*N*m, number of migrants per generation) using GenAlEx v6.503 (Peakall and Smouse, 2006), using a matrix of squared Euclidean distances computed from individual multilocus phenotypes. The AMOVA calculated by GenAlEx calculates Phi-statistics (PHI_{PT}) as an analogue to the traditional F_{ST} and estimates variation among regions, variation among populations between regions, and variation among individuals within populations. Phi-statistics is a modified version of Wright's that refers to

the relative contributions of between-population separation to the overall genetic variation in the whole sample, the greater the F_{ST} or PHI_{PT} values are, the greater the differences between populations. Genetic differentiation was assessed by comparing the average number of pairwise differences among and within populations; the higher these values are, the higher the differences between populations.

2.4.6 Bayesian Phylogenetic Trees

I conducted Bayesian phylogenetic analyses to find evidence of systematic relationships within sampling sites for cyt-b and CR sequence alignments. I constructed phylogenetic trees with MrBayes Geneious Plugin v.3.2.6 (Huelsenbeck and Ronquist, 2001). I determined the best model for nucleotide substitution using the Akaike Information Criterion (AIC) in JModeltest v2.1.1 (Guindon and Gascuel, 2003; Darriba et al., 2012), which supported the Jukes-Cantor (JC) model as the best fit; this model assumes that the substitution of a base with any other occurs with equal probability (Erickson, 2010). MrBayes ran with default settings (gamma rate variation, N_{st} of 2, MCMC chain length = 1,100,000, sample freq = 200, heated chains= 4, and burn-in length=100,000). The relevant region of the mitochondrial genome of *A. jamaicencis* (AF061340.1) was used as an outgroup to root the trees. The trees were exported as Nexus files and edited in FigTree v.1.4.3 (Rambaut, 2012).

2.5 Results

2.5.1 Species Identification

The BLAST search for both cyt-b and Control Region confirmed the taxonomy of the species with all consensus sequences matching by >95% to the NCBI database of *Leptonycteris yerbabuenae* (previously known as *Leptonycteris curasoae yerbabuenae*).

Cytochrome b Analysis

2.5.2 Mitochondrial cyt-b haplotype diversity patterns

After trimming and filtering, the dataset for cyt-b from 57 sequences with a length of 306 bp. I included 119 additional sequences of the same length from Arteaga (2018) for a total of 170 sequences. The dataset described 14 unique mtDNA haplotypes (h) defined by 16 variable sites (S), and corresponding to 11 singletons and 5 parsimony-informative sites. Overall, diversity levels for cyt-b were low with average values of nucleotide diversity of π = 0.0009 and haplotype diversity Hd=0.241 (Table 2.1). The number of individuals in each group varied from 2 to 145 with the majority of the samples represented in haplotype 2.

Table 2.1 Genetic diversity of mitochondrial cyt-b of *Leptonycteris yerbabuenae*. Sample size (N), polymorphic sites (S), number of haplotypes (h) with total in bold; haplotype diversity (Hd), average number of differences (k), and nucleotide diversity (π) with average in italics.

			cyt-b (306 bp)				
Region	**Colony**	**N**	**S**	**h**	**Hd**	**K**	**π**
	Baja		1				
northern	California	119	2	11	0.291	0.375	0.00117
northern	Pinacate	17	1	2	0.117	0.117	0.00037
central	Colima	18	2	3	0.215	0.222	0.00069
southern	Chiapas	16	3	3	0.341	0.483	0.00151
			1				
	Total	**170**	**6**	**14**	*0.241*	*0.299*	*0.00093*

2.5.3 Test of neutrality and estimates of population expansion

Neutrality tests showed evidence of recent population expansion (Tajima's test D: -2.222 ($p<0.01$**), where $D < 0$ suggests population expansion or purifying selection). Fu's Fs statistic was -14.595 (a negative value is evidence for an excess number of alleles as expected for a recent population expansion or from genetic hitchhiking). Fu and Li's D* test statistic was -4.062 ($p<0.02$**) and Fu and Li's F* test statistic -4.039 ($p<0.02$**; negative values indicate an excess of singletons). The mismatch distribution analysis showed a positively skewed unimodal distribution that can be interpreted as indicative of very sudden population expansion (Figure 2.3).

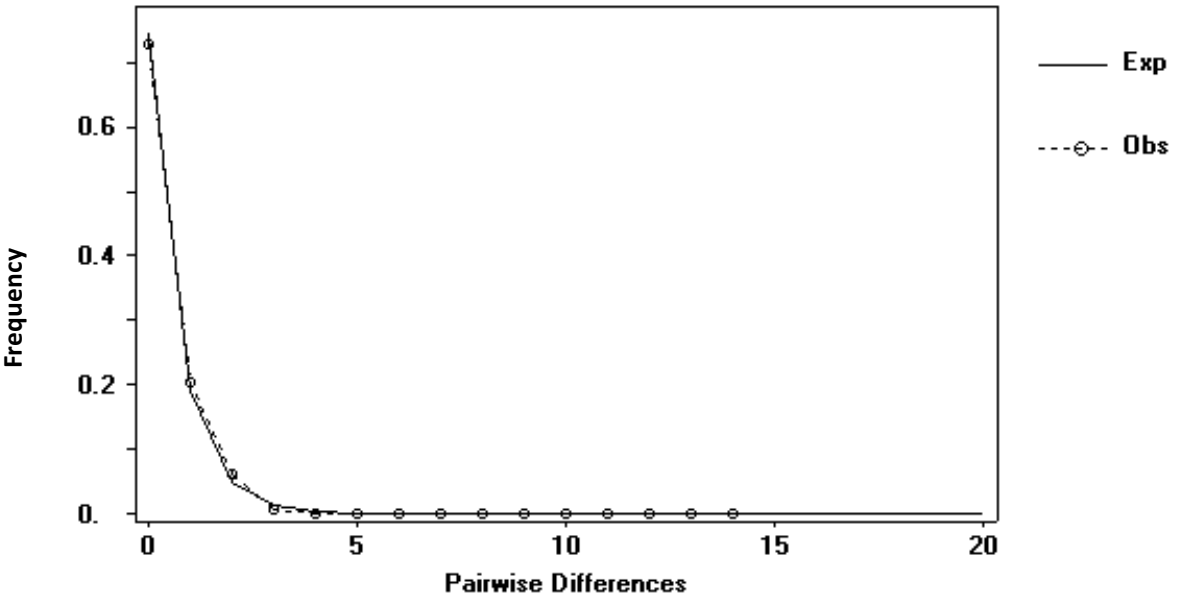


Figure 2.3 Mismatch distribution of pairwise differences based on cyt-b of *L. yerbabuenae* from Baja California, Pinacate, Colima and Chiapas. Harpending's raggedness index as calculated by DnaSP (r=0.09, p<0.00001). Solid line: expected values (Exp); dotted line: observed values (Obs), under a sudden population expansion model.

2.5.4 Species diversification time

With the cyt-b dataset, I estimated the species divergence time to occur at some point between the late Pleistocene and beginning of the Holocene (0-1 mya; Figure 2.4). This estimate is in accordance with previous calculations of phyllostomid diversification (Datzmann et al., 2010, Baker et al., 2019), and that of *L. yerbabuenae* as predicted by Arteaga et. all (2018). The Last Glacial Maximum (~23-18 kyr) promoted the genetic diversity of several Neotropical species (Ramírez-Barahona and Eguiarte, 2013), including several bat species (see: Dixon 2011; Stoffberg et al. 2012; Bilgin et al. 2016), and desert plant species (Cornejo-Romero et al., 2017; Clark-Tapia and Molina-Freaner, 2003) that may have increased food resources available for *L. yerbabuenae.*

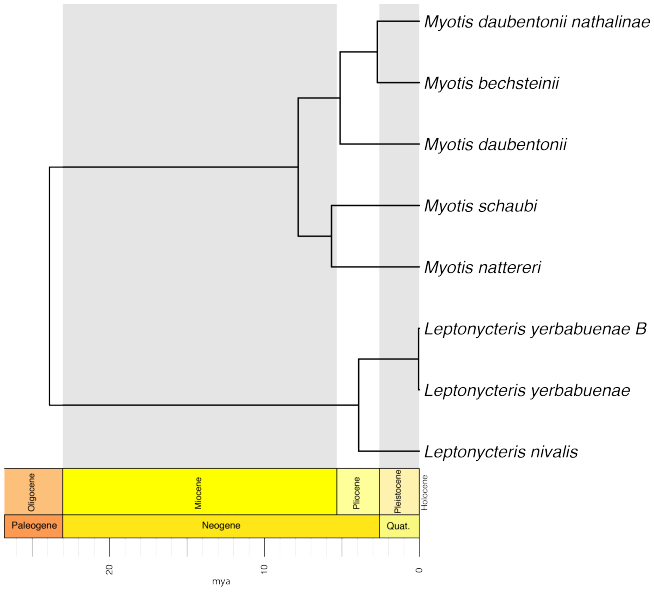


Figure 2.4 Bayesian dating of *Leptonycteris yerbabuenae* diversification based on cyt-b.

2.5.5 Statistical parsimony haplotype network

The statistical parsimony haplotype network for cyt-b (Figure 2.5) presents a star-like shape, which suggests that *L. yerbabuenae* experienced a bottleneck followed by population

expansion. One main haplotype (H2) represented 85.6% of all samples with 145 individuals (99 from Baja California, 14 from Chiapas, 16 from Colima and 16 from Pinacate; Table 2.2) and could be distinguished by 13 other haplotypes by only one or two mutations (Figure 2.5). A similar star-like pattern is observed with sequences from Colima, Chiapas and Pinacate (Figure 2.6). Pairwise population comparisons of genetic differentiation (Φ_{ST}) did not reveal a clear differentiation pattern between the northern (Baja California and Pinacate), central (Colima) or southern (Chiapas) samples (Φ_{ST} = 0.035, p=0.128) (Figure 2.1). A Φ_{ST} value of 0 indicates no differentiation between populations, whereas a value of 1 would indicate complete differentiation. Most of the samples were included in only one haplotype with 1-2 mutations (Figure 2.5); the levels of sequence divergence constitute <2% of the total variation.

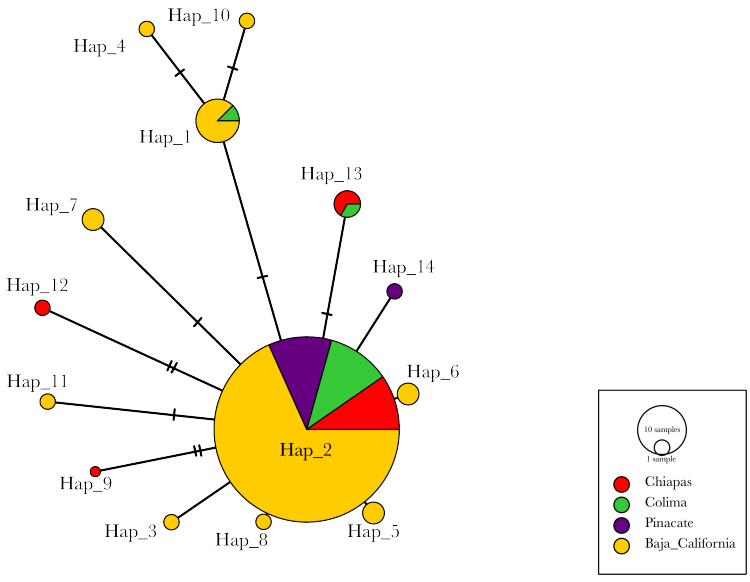


Figure 2.5 Statistical parsimony haplotype network based on mitochondrial cyt-b. Circle size is proportional to haplotype frequency; connective lines show the number of mutational steps as hatch marks, (N=170; Chiapas=17, Colima=18, Pinacate=17, Baja California=119).

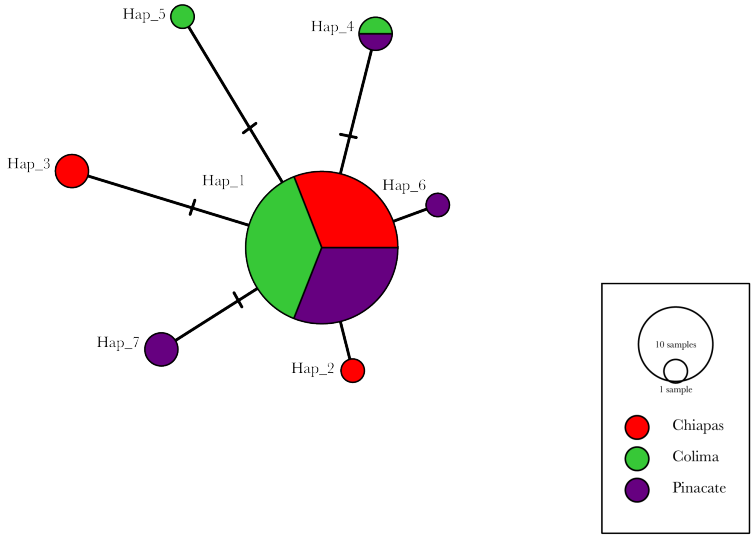


Figure 2.6 Statistical parsimony haplotype network based on mitochondrial cyt-b. Circle size is proportional to haplotype frequency; connective lines show the number of mutational steps as hatch marks, (N=51: Chiapas= 16, Colima=18, Pinacate=17).

Table 2.2 cyt-b haplotypes identified in 170 samples of *L. yerbabuenae* populations from Chiapas, Colima, Pinacate and Baja California.

	Number of samples				
Haplotype No.	**Chiapas**	**Colima**	**Pinacate**	**Baja California**	**Total**
1	0	1	0	7	8
2	14	16	16	99	145
3	0	0	0	1	1
4	0	0	0	1	1
5	0	0	0	2	2
6	0	0	0	2	2
7	0	0	0	2	2
8	0	0	0	1	1
9	0	0	0	0	0
10	0	0	0	1	1
11	0	0	0	1	1
12	1	0	0	0	1
13	2	1	0	0	3
14	0	0	1	0	1

2.5.6 Analysis of Molecular Variance (AMOVA)

The results from the AMOVA indicate that most of the variation (>98%) was found within populations and did not detect significant differences among Chiapas and Baja California (PHI_{PT}=0.039, p=0.054), and Chiapas and Pinacate (PHI_{PT}=0.030, p=0.103). On the other hand, the negative values shown between the rest of the comparisons revealed no significant differences which could be indicative of higher genetic exchange between those populations (Table 2.3). As values of PHI_{PT} and Φ_{ST} are equal (0.03) we can assume that all loci have equal, additive and independent effects and that population divergence is driven only by neutral genetic drift (Edelaar et al., 2011).

Table 2.3 Pairwise population estimates between sampling localities based on cyt-b mitochondrial DNA *of L. yerbabuenae*. PHI_{PT} values below diagonal. Probability, P(rand >= data) based on 9999 permutations is shown above diagonal.

	Baja California	**Chiapas**	**Colima**	**Pinacate**
Baja California		0.054	0.396	0.384
Chiapas	0.039		0.230	0.103
Colima	-0.019	-0.014		0.485
Pinacate	-0.004	0.030	-0.001	

2.5.7 Bayesian Phylogenetic Tree

The Bayesian phylogenetic inference tree (Supplementary Figure 2.11) recovered one clade with posterior probability of 1, composed of an admixture of haplotypes from all four regions (Baja California, Colima, Chiapas and Pinacate), suggesting panmixia in *L. yerbabuenae* and explained by low genetic diversity between haplotypes. Two other clades (pp>8) were composed of 2 samples from the same origin and one composed of 2 samples form Chiapas and 2 from Pinacate. A fifth clade (pp = 0.77) contained 9 samples from Baja California and 1

from Colima. A Neighbour-Joining tree shows the relationship of the cyt-b haplotypes (Figure 2.7) and further confirms that panmixia is likely to explain the non-structuring of *L. yerbabuenae*.

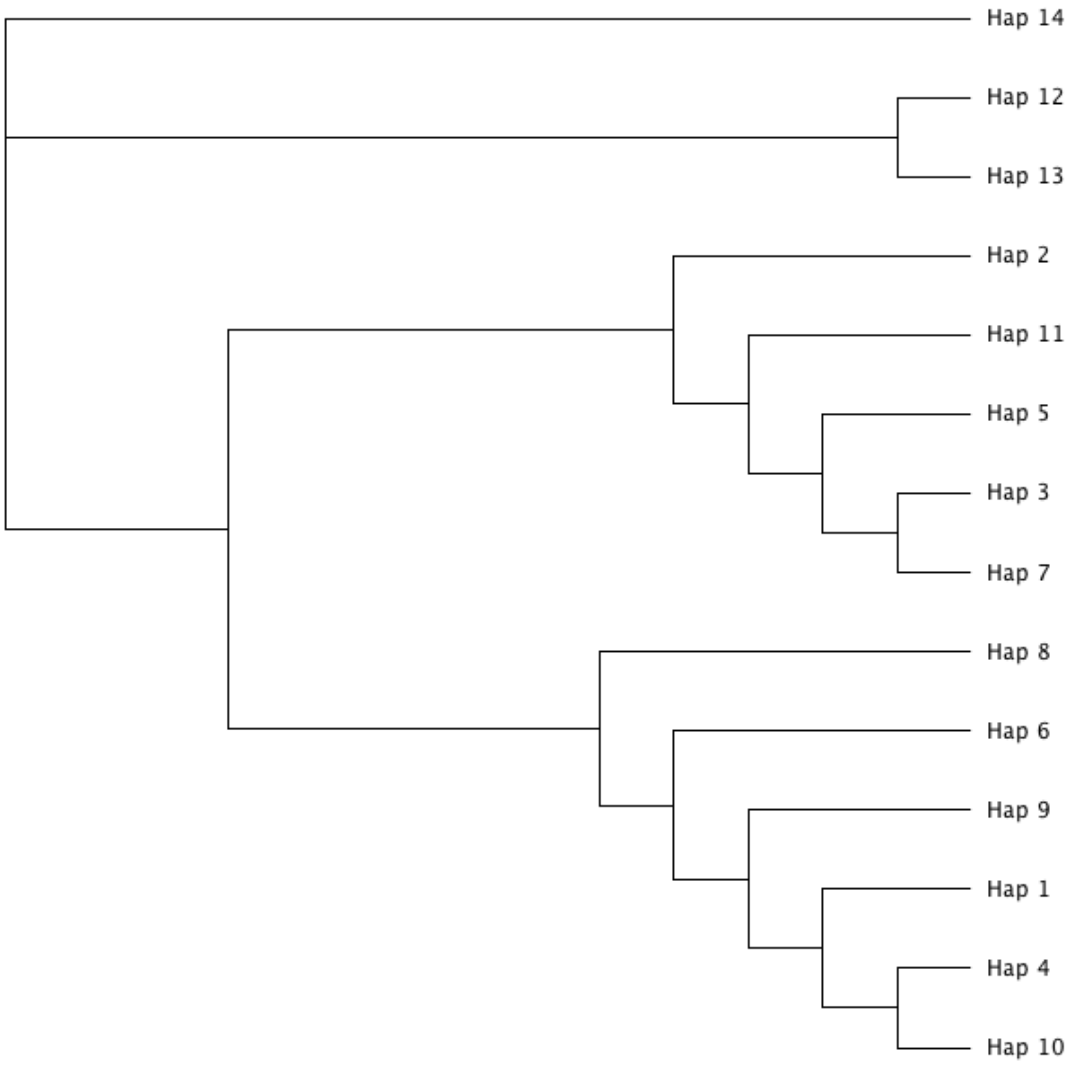


Figure 2.7 Neighbour-Joining phylogenetic tree of mitochondrial cyt-b haplotypes constructed under the HKY distance model as implemented in Geneious.

Control Region Analysis

2.5.8 Mitochondrial CR haplotype diversity patterns

The dataset of CR contained 57 sequences with a length of 497 bp corresponding to 28 unique mtDNA haplotypes (h) defined by 26 variable sites and corresponding to 10 singletons and 16 parsimony-informative sites. The results obtained for cyt-b reflect moderate levels of haplotype diversity with average values of Hd=0.873 and low levels of nucleotide diversity with average values of π = 0.008 (Table 2.4). The number of haplotypes in each group varied from 2 to 15, and the majority of the samples were included in haplotype 1 (Table 2.5).

Table 2.4 Genetic diversity of mitochondrial CR of *Leptonycteris yerbabuenae*. Sample size (N), polymorphic sites (S), number of haplotypes (h) *with total in bold;* haplotype diversity (Hd), average number of differences (k), and nucleotide diversity (π) with average in italics.

		Control Region (D-loop, 497 bp)					
Region	**Colony**	**N**	**S**	**h**	**Hd**	**K**	**π**
northern	Pinacate	19	15	12	0.935	3.730	0.00751
central	Colima	18	19	15	0.980	4.692	0.00944
southern	Chiapas	20	14	9	0.705	3.315	0.00667
		57	**26**	**28**	*0.873*	*3.913*	*0.00787*

2.5.9 Test of neutrality and estimates of population expansion

Neutrality tests also showed evidence of recent population expansion: Tajima's test D: -1.028 (p>0.1 NS). Fu's Fs statistic: -17.711, Fu and Li's D* test statistic: -1.338 (p>0.1 NS), Fu and Li's F* test statistic: -1.460 (p>0.10 NS). The mismatch distribution analysis showed a bimodal distribution (Figure 2.8), which is characteristic of species under the influence of a strong biogeographic barrier and can be indicative of the presence of two divergent mitochondrial lineages (Gaither et al., 2011). Furthermore, distributions are usually multimodal in samples

drawn from populations at demographic equilibrium or in decline (Dadi et al., 2012; Joshi et al., 2013).

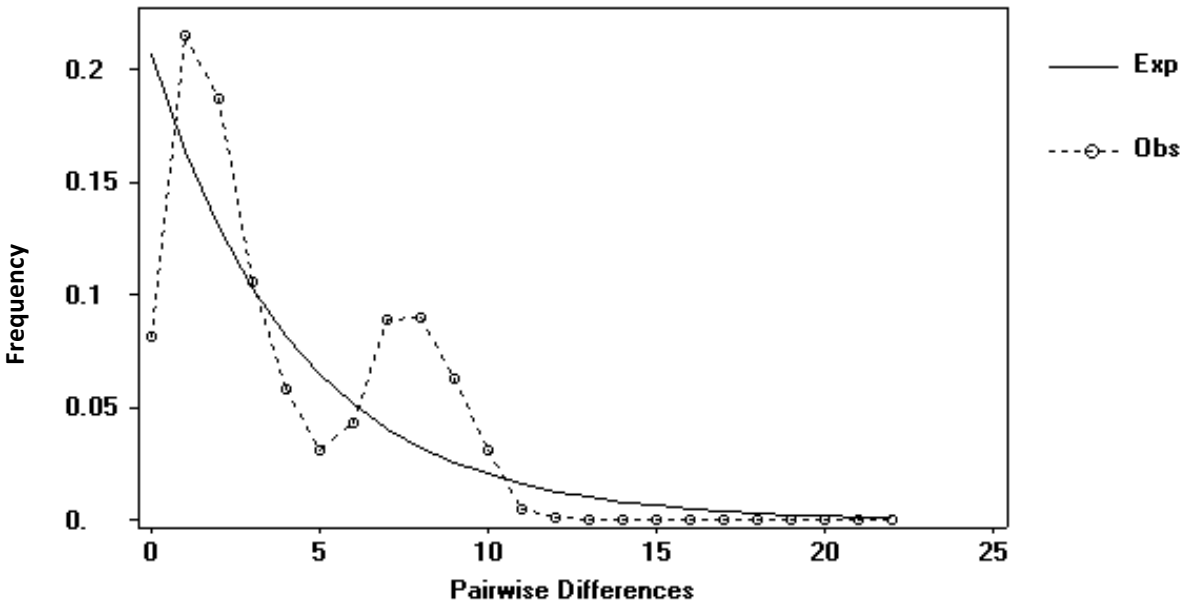


Figure 2.8 Mismatch distribution of pairwise differences based on CR of *L. yerbabuenae* from Pinacate, Colima and Chiapas localities. Harpending's raggedness index as calculated by DNAsp (r=0.08189, p<0.00001). Solid lines: expected values (Exp); dotted lines: observed values (Obs), under sudden population expansion model.

2.5.10 Statistical parsimony haplotype network

The statistical parsimony haplotype network for CR (Figure 2.9) also presents a star-like pattern with a common haplotype surrounded by many singletons. One main haplotype (H1) represented 26% of all samples with 15 individuals (11 from Chiapas, 1 from Colima, and 3 from Pinacate) and could be distinguished by 27 other haplotypes by one to six mutations. Most of the samples from Chiapas were assigned to haplotype 1, while 19 were unique (Table 2.5). Pairwise Φ_{ST} population comparisons did not reveal a differentiation pattern among Pinacate, Colima or Chiapas samples (Φ_{ST} = 0, p<0.001). A Φ_{ST} value of 0 indicates no

differentiation between populations, whereas a value of 1 would indicate complete differentiation.

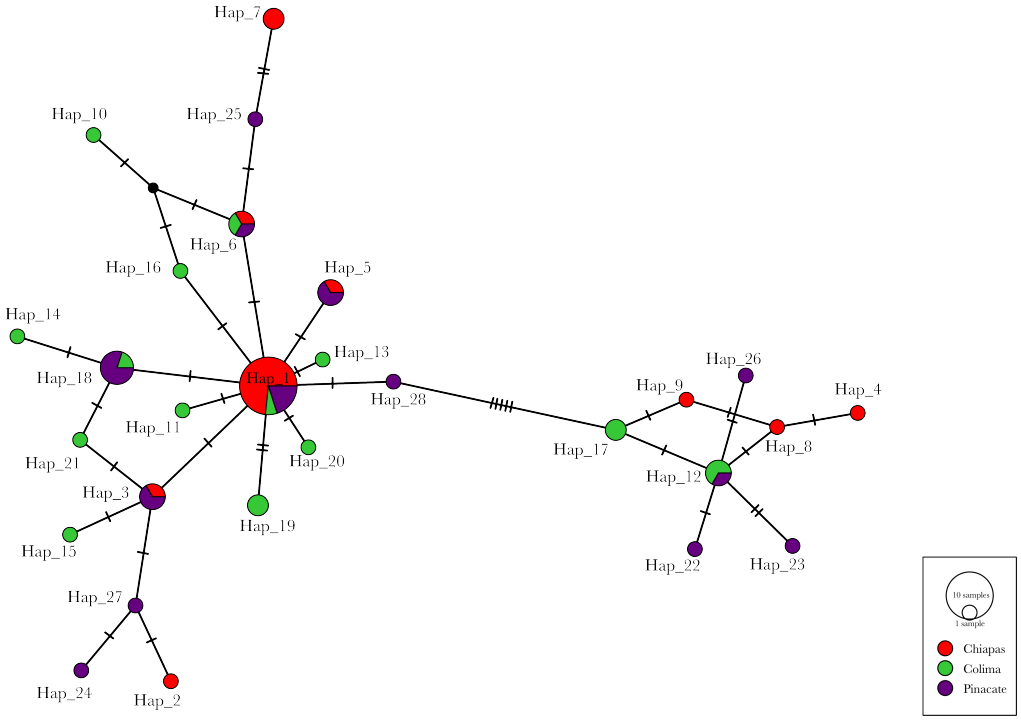


Figure 2.9 Statistical parsimony haplotype network based on mitochondrial Control Region (CR) of *L. yerbabuenae*. Circle size is proportional to haplotype frequency; connective lines show the number of mutational steps as hatch marks, N=57.

Table 2.5 Control Region haplotypes identified in 57 samples of *L. yerbabuenae* populations from Chiapas, Colima and Pinacate

Haplotype No.	Number of samples			
	Chiapas	Colima	Pinacate	Total
1	11	1	3	15
2	1	0	0	1
3	1	0	2	3
4	1	0	0	1
5	1	0	2	3
6	1	1	1	3
7	2	0	0	2
8	1	0	0	1
9	1	0	0	1
10	0	1	0	1
11	0	1	0	1
12	0	2	1	3
13	0	1	0	1

14	0	1	0	1
15	0	1	0	1
16	0	1	0	1
17	0	2	0	2
18	0	1	4	5
19	0	2	0	1
20	0	1	0	2
21	0	1	0	1
22	0	0	1	1
23	0	0	1	1
24	0	0	1	1
25	0	0	1	1
26	0	0	1	1
27	0	0	1	1
28	0	0	1	1

2.5.11 Analysis of Molecular Variance (AMOVA)

The results from the AMOVA indicate that most of the variation (>98%) was found within populations but did not detect any significant differences between the southern group in Chiapas, the central group in Colima or the northern group in Pinacate (Table 2.6).

Table 2.6 Pairwise population estimates between sampling localities based on CR mitochondrial DNA of *L. yerbabuenae*. PHI_{PT} values below diagonal. Probability, P(rand >= data) based on 9999 permutations is shown above diagonal.

	Chiapas	**Colima**	**Pinacate**
Chiapas		0.324	0.340
Colima	-0.012		0.311
Pinacate	-0.025	-0.022	

2.5.12 Bayesian Phylogenetic tree

The Bayesian phylogenetic inference tree (Supplementary Figure 2.12) shows that one clade with posterior probability of 1 contained the majority of the samples and was composed of an admixture of haplotypes from all three regions (Colima, Chiapas and Pinacate) suggesting panmixia in *L. yerbabuenae.* A second smaller clade with posterior probability of 1 also grouped samples from the tree regions. Two other clades (pp>0.9) contained samples from

Chiapas and Pinacate and one clade (pp=0.9) contained samples from Colima. A Neighbour Joining tree shows the relationship of the CR haplotypes (Figure 2.10).

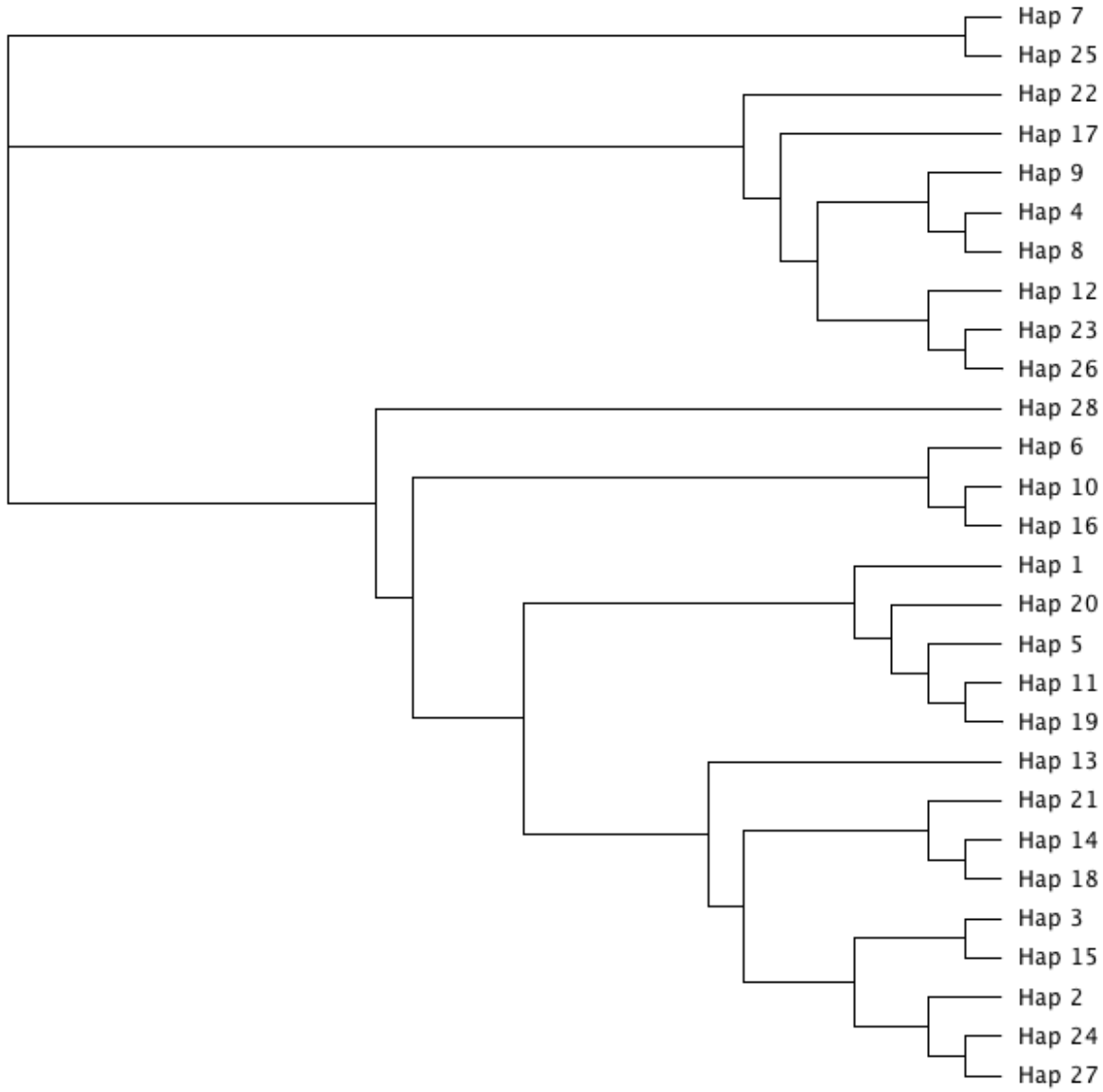


Figure 2.10 Neighbour-Joining phylogenetic tree of mitochondrial control region haplotypes constructed under the HKY distance model as implemented in Geneious.

2.6 Discussion

This is a comprehensive population genetics study of *L. yerbabuenae* in three localities in Mexico based on two mitochondrial DNA regions. The data show that the three populations

correspond to the species *Leptonycteris yerbabuenae,* which exhibits partial migration with a northern female population that performs a long-distance migration of > 1700 km from the centre to northern Mexico and a southern female population performing seasonal altitudinal migrations. Males remain yearlong in the centre of Mexico and are also considered a resident population. I generated 51 partial sequences for the mitochondrial cyt-b gene (306 bp) and 57 partial sequences for the mitochondrial Control Region (497 bp). These data contribute additional information for *L. yerbabuenae* in its most southern range (Figure 2.1). With it, I present new and relevant information to the understanding of the evolutionary history patterns of genetic partitioning pertinent to the ecology and migratory behaviour of *L. yerbabuenae.*

Previous studies on population genetics of *L. yerbabuenae* have found moderate levels of genetic differentiation between northern and south-central populations across its range (Morales-Garza et al., 2007; Ramirez, 2011). Here, I tested this hypothesis in three populations distributed in the most northern and southern parts of Mexico and the centre. The analysis of mitochondrial cyt-b and CR haplotype distribution allowed me to identify the presence of high-frequency haplotypes common to all colonies. The link of one common haplotype with others in lower frequencies (singletons) is a pattern often attributed to populations that have experienced recent expansion (Slatkin and Hudson, 1991). The results show that the data do not describe a clear pattern of population structure across sampling locations.

In general, migratory bat species are expected to exhibit little intraspecific genetic structuring even over large geographic distances (Moussy et al., 2013; McCracken and Gassel, 1997) The

ability to fly over large distances in a single night seems to facilitate significantly extensive gene flow among populations (Russell and McCracken, 2006). Moreover, the potential for long-distance dispersal and large population sizes may generate low levels of genetic subdivision that will eventually create panmictic populations (Russell and McCracken, 2006; Sovic et al., 2016).

The analysis for mitochondrial cyt-b gene revealed low levels of genetic diversity within northern individuals in Baja California and Pinacate, central individuals in Colima and southern individuals in Chiapas with almost all individuals allocated to one common haplotype (Table 2.2). The AMOVA analysis indicates no significant genetic variation between Chiapas and Baja California (p=0.05) (Table 2.3). The significant neutral tests, the unimodal mismatch distribution (Figure 2.3) and the star-shaped haplotype network (Figure 2.5) support the hypothesis of recent population expansion. Although the Bayesian phylogenetic tree subdivides the populations into several clades, these contain individuals from all localities (Supplementary Figure 2.11).

The analysis for mitochondrial Control Region revealed moderate levels of genetic diversity within northern individuals in Pinacate, central individuals in Colima and southern individuals in Chiapas with almost all individuals allocated to one common haplotype but a high number of unique haplotypes (Table 2.4). The AMOVA analysis did not indicate any significant variation among localities (Table 2.6). The significant neutrality tests and the star-shaped haplotype network surrounded by many unique haplotypes (Figure 2.9) support the hypothesis of recent population expansion. Furthermore, the bimodal mismatch distribution based on the hyper variable fragment of the CR (Figure 2.8) indicates populations at

demographic equilibrium, which is common in species under the influence of a strong biogeographic barrier and suggests the presence of two divergent mitochondrial lineages. Furthermore, multiple haplo-groups within a single interbreeding population that show a sequence divergence of 3% or more occur when relatively large populations are maintained over many generations thus allowing an equilibrium between mutation rate and drift leading to high levels of genetic diversity (Morgan-Richards et al., 2017). Although the Bayesian phylogenetic tree subdivides groups the samples in to several clades, these and contain individuals from all localities thus failing to reveal differentiation (Supplementary Figure 2.12).

The slightly higher levels of variation in CR than cyt-b is explained by the different evolutionary rates of each gene; the Mitochondrial Control Region contains variable blocks that evolve 4-5 times faster than that of the entire mtDNA molecule, thus when studying conspecific populations, this region provides better resolution (Smith and Wayne, 1997). However, both mitochondrial markers show a consistent pattern of genetic diversification and support on the hypothesis of an un-differentiated population living in northern and central Mexico; the results also demonstrate that the population in the south maintains matrilineal genetic flow with the northern and central groups.

Overall genetic differentiation at the mtDNA level was not significant to distinguish population structure among sampled colonies of *L. yerbabuenae*. However, the slight higher PHI_{PT} values obtained with the AMOVA for cyt-b could be the result of a recent split between the southern group in Chiapas and the northern group in Baja California and Chiapas and the northern group in Pinacate and could be the result of the influence of physical barriers to migration and historical population demography caused by climatic oscillation during the

Pleistocene or Holocene (Arteaga et al., 2018). Furthermore, the bimodal mismatch distribution obtained for CR could be evidence of two mitochondrial lineages.

High-frequency haplotypes are likely to be old alleles with broad geographical distribution as their carriers have probably had relatively long time to disperse. The haplotypes with only one connection (singletons) are considered relatively recent and together create a star-like pattern of networks, which can be interpreted as rapid population expansion, consistent with the results of the neutrality tests.

These findings agree with previous genetic studies where patterns of genetic differentiation were found between samples from central and northern Mexico (Ramirez, 2011; Morales-Garza et al., 2007) and show for the first time that the population in southern Mexico is not significantly different to the ones in central and northern Mexico. Both mitochondrial markers show very low levels of population structure within the studied populations, which is consistent with previous phylogeographic studies of *L. yerbabuenae* (Wilkinson and Fleming 1996; Morales-Garza et al. 2007; Ramirez 2011).

However, the results obtained here failed to support the results found by Morales-Garza (2007) using nuclear RAPD markers and those of Ramirez (2011) using microsatellite loci, which differentiate more clearly the northern population from the south-central population and because these markers are inherited biparentally they were also useful to compare the dispersal patterns of males versus females and to infer philopatry. However, the suite of genetic differentiation estimators used in my study suggests a deep matrilineal phylogenetic convergence between all populations and only weak genetic differentiation was observed

between the southern group in Chiapas and the northern group in Baja California and agree with the findings of Arteaga (2018) and the analysis of mitochondrial CR of Ramirez (2011).

Genetic structure is influenced by population size, sex-biased dispersal and mating patterns. If males disperse more than females, which is the case of *L. yerbabuenae* (Wilkinson and Fleming, 1996; Arteaga et al., 2018), the genetic structure may be less strongly correlated with the geographic distribution observed than by screening diploid loci such as RAPDs or microsatellite loci. Furthermore, mtDNA introgression can occur between conspecific populations and can potentially hide real phylogenetic relationships; for these two reasons,

I do not discard the hypothesis of two clades, one in the north and another in the south as proposed by Morales-Garza (2007). Future analysis with nuclear markers, Restriction site Associated DNA Sequencing (RAD-seq) or Whole Genome Sequencing (WGS) will be useful to resolve more subtle patterns of population subdivision within the south-central population and will further clarify subtle population genetic structure in this region related to life history variations. It will be exciting to use genomics in the future to further differentiate the northern migratory population from the south-central non-migratory populations and test the hypothesis that clear population structure exists and can be detected with WGS.

The widespread distribution and migratory habits of *L. yerbabuenae* mean that it not only has the ability to exploit diverse niches, ranging from deserts to lowlands and tropical forests but that it can be very vulnerable to sudden environmental and climate changes that have an adverse effect on the resources it depends on at specific times of the year. My results have important implications for the management and conservation of *L. yerbabuenae,* particularly

in its most southern range. Although annual monitoring of the populations in the north has shown a positive increase, the colonies in the south have not received much attention in recent years. In particular, the cave Los Laguitos in Chiapas (southern Mexico; Fig. 1.4) is located in private lands which are not monitored, and is very close to the main highway. During my visits, I found evidence of vandalism inside the cave, and huge amounts of rubbish. The cave is situated roughly at 6 km from the National Park Canon del Sumidero but within the boundaries of Tuxtla Gutierrez City. This cave is the only maternity roosting site within the area and harbours more than 5,000 individuals.

The percentage of sequence divergence for both mitochondrial markers used in this study is less than 2% and is indicative of typical levels of intraspecific variation as proposed by Bradley & Baker (2001). Although it is controversial to use these percentage to make assumptions about species demarcation (Vogler and Desalle, 1994), I found them useful to treat the populations in southern Mexico as a different ESU for its difference in migratory behaviour, timings of parturition and the different ecosystem it inhabits.

As a migratory species, *L. yerbabuenae* has the advantage of finding better quality resources and more suitable habitats but faces many challenges along its route. Its ability to adapt to a diverse diet and changing environmental conditions could be evidenced by changes in aspects such as their microbiome and immune system. Adapting to a new environment may not only alter their genetics, but phenotypic adaptations also are detected if the species undergoes selection to exploit resources better; this will be explored in the next chapter.

2.7 Supplementary Figures

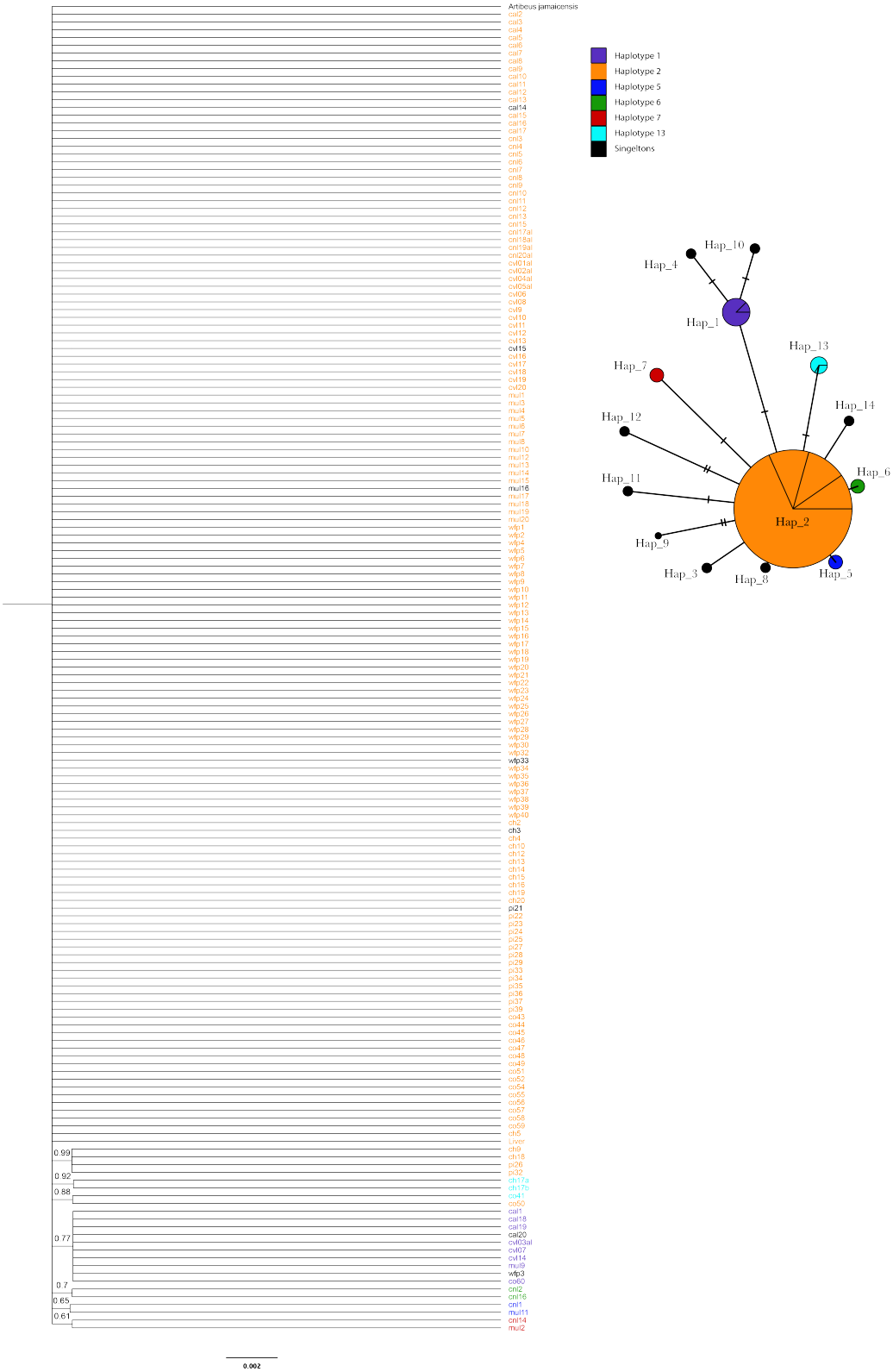


Figure 2.11 Cladogram inferred based on analysis of cyt-b sequences with MrBayes. Support values (Posterior Probabilities) are on top of branches. Values of 1 are not presented. Samples names from Chiapas start with “ch”, from Pinacate with “pi” and from Colima with “co” and “Liver”, and Baja California with “wfp”, “cal”, “cnl”, “mul”, “cvl”. Haplotype codes are colour coded. *Artibeus jamaicensis* was used as outgroup to root the tree.

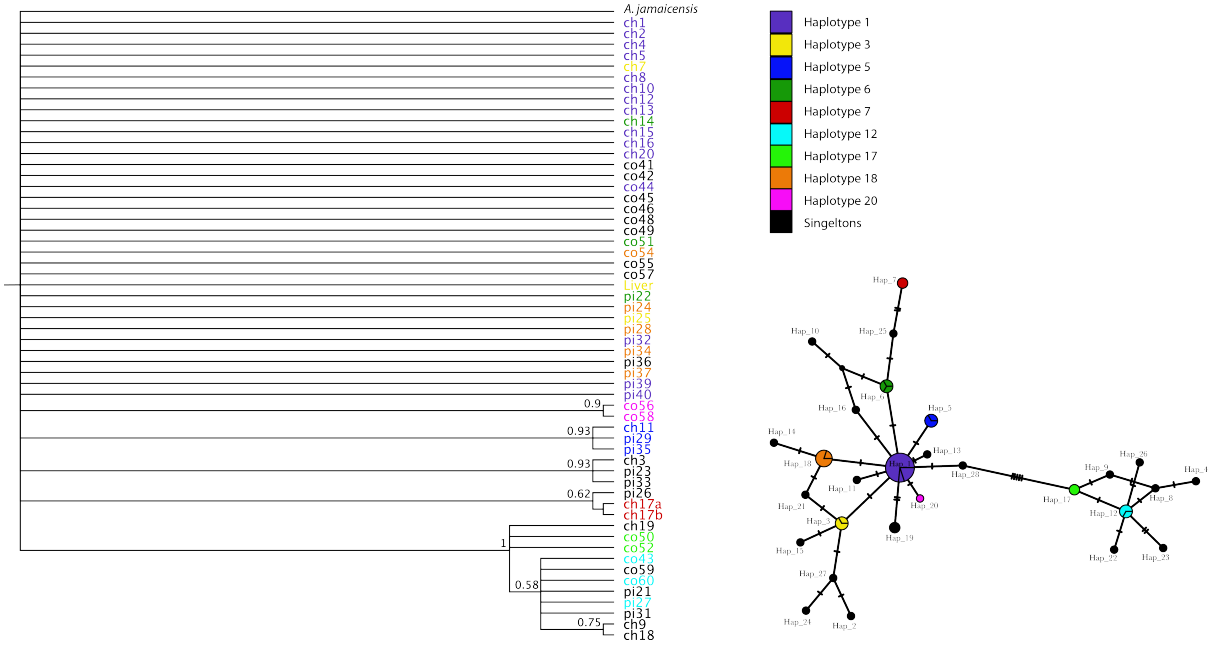


Figure 2.12 Cladogram inferred based on analysis of control region sequences with MrBayes. Support values (Posterior Probabilities) are on top of branches. Values of 1 are not presented. Samples names from Chiapas start with “ch”, from Pinacate with “pi” and from Colima with “co” and “Liver”. Haplotype codes are colour coded. *Artibeus jamaicensis* was used as outgroup to root the tree.

Chapter 3. Ecological morphology and wing adaptations for migration in the tequila bat (*Leptonycteris yerbabuenae*)

Abstract

Wing shape is an informative predictor of flight performance in flying animals and can be used to infer ecological traits such as habitat use and migratory ability. Here I used traditional morphometric analyses of 13 linear measurements and geometric morphometric analyses of 15 landmarks to reveal significant variation in wing traits across three populations of *Leptonycteris yerbabuenae* with different migratory strategies (migratory females from northern Mexico, non-migratory females from southern Mexico, and non-migratory males from central Mexico). The data unveiled signatures of dimorphism associated with geographical variability in wing size and shape related to migratory behaviour and foraging strategies.

Sexual dimorphism has been found in wing and body characters of bats and explained as the result of adaptive pressures that load carrying has on females during reproduction. This is the first study to show that wing shape changes occur at the population level as a result of different environmental pressures due to migration. My findings suggest that the wings of migratory females have a more aerodynamic shape with narrower wings as a result of higher aspect ratio for flight endurance and higher wing loading for fast flight than the non-migratory bats. Non-migratory males and non-migratory females have wings with profiles better suited for hovering, gleaning and manoeuvring in dense vegetation (e.g. lower aspect ratio, more rounded wing tips, and lower wing loading) compared with bats from migratory populations. The variation in wing shape is broadly explained by differences in the length of the metacarpals and in the hand-wing area relative to the rest of the wing.

3.1 Introduction

3.1.1 Wing morphology and flight performance

The aerodynamic performance of bats has been correlated to morphological traits including wing loading (body mass per unit of wing area), aspect ratio (ratio of wing length to width), tip length ratio (length of wing to arm), tip area ratio (area of chiropatagium to plagiopatagium), and wing tip shape (overall size and shape) (Figure 3.1). These wing shape traits affect measures of flight performance such as flight speed and manoeuvrability, hence links occur between wing morphology, flight performance and habitat use (Norberg and Rayner, 1987).

One of the classic observations of the relationship between the ecology of an individual and its morphological adaptations is that long-distance migrants tend to have more acute wings than more sedentary individuals; wing pointedness results in a high aspect ratio allowing more energy efficient and faster flight that reduces drag compared to wings with more rounded tips (Monkkonen, 1995; Bahlman et al., 2006). This prediction has been tested numerous times at the species level (Norberg and Rayner, 1987; Norberg, 1981; Rhodes, 1995; Aldridge and Rautenbach, 1987). Furthermore, aerodynamic models predict the optimal wing shape for bats adapted for different flight modes (Norberg and Rayner, 1987); in long-distance migrants, natural selection favours shapes that contribute to economic forward flight at high speeds such as high wing loading and high aspect ratio (Aldridge and Rautenbach, 1987).

In contrast, animals that fly in cluttered environments have shorter wingspans, lower wing loading and lower aspect ratios that contribute to lower speed flights which in turn allows

better hovering and manoeuvrability in dense vegetation (Norberg and Rayner, 1987; Aldridge and Rautenbach, 1987); furthermore, females have larger wing areas, which allows them to counteract the effects of carrying additional weight on flight performance (Hayssen and Kunz, 1996).

Wing measurements have been used as proxies for flight efficiency and speed, and have been used to quantify the relationship between mobility and occupancy across different habitats and the degree of susceptibility of bat species to habitat changes (Bader et al., 2015; Marinello, 2014). Wing morphology has also been associated with foraging strategies in insectivorous bats as an adaptive mechanism underpinning resource partitioning (Mancina et al., 2012; Mojmir Sevick, 2003). Differences in wing shape have even been used to differentiate migratory bird subspecies from sedentary ones that overwinter sympatrically (Copete et al., 1999). Furthermore, studies on bird wing morphology confirm an evolutionary relationship between wing shape and migration in non-passerine birds (Minias et al., 2015), and associate flight efficiency with morphological adaptations in birds with different flight mechanisms (Grilli et al., 2017).

Although sexual dimorphisms has been found in migratory behaviour and body size in the common noctule bat (*Nyctalus noctula*), variation related to shape has not been detected between migratory females and sedentary males (O'Mara et al., 2016). More studies on bat wing allometry related to flight performance and ecological morphology are necessary to show that variation in migratory behaviour at population level is consistent with aerodynamic theory.

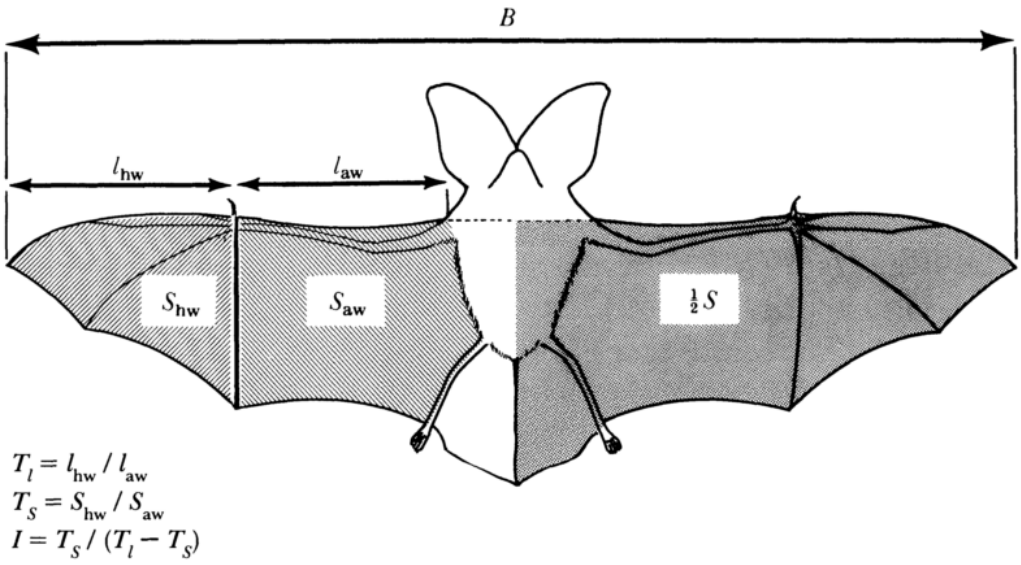


Figure 3.1 Wing Ratios: wingspan (B), wing area (S), area of hand-wing (Shw), area of arm-wing (Saw), length of hand-wing (Lhw), length of arm-wing (Law). Tip length ratio (Ti), Tip area ratio (Ts), wingtip shape index (I). From Norberg & Rayner (1987).

3.1.2 Using geometric morphometrics to study wing shape variation

Differences in wing shape between migratory and non-migratory populations can be further explored with the use of geometric morphometrics. This approach allows the identification of very subtle variation among groups and is a potent tool to analyse variation in shape and the identification of its causes; the key advance of geometric morphometrics by comparison to traditional morphometrics is that it uses the complete information about a configuration of landmarks and all the interrelations among them and hence not only uses a selected set of linear distances (Klingenberg, 2016).

Geometric morphometrics has been used extensively to study variation in wing morphology of insects (see: Sadeghi et al. 2009; Kandemir et al. 2011; Outomuro et al. 2013; Leandro et al. 2016). The approach uses data to quantify shape as landmark coordinates, outline curves and surfaces. With this method, it is possible to apply Cartesian coordinates to quantify

variation in the shape of anatomical objects (Adams et al., 2013). The studies are based on the *Procrustes paradigm*, an analytical procedure that uses superimposition of landmark configurations to obtain shape variables (Adams et al., 2013).

Although geometric morphometrics has proven to be a very powerful tool to study wing variation its application in the study of wing shape of bats has not been as widespread as the numerous studies on insects, especially to compare migratory versus non-migratory populations. To my knowledge, only three studies have applied geometric morphometrics to study bat wings. The first study was used to detect subtle sexual dimorphism in the little yellow-shouldered bat (*Sturnira lilium*) suggesting different flight patterns between males and females; the study identified different area distributions in distinct portions of the wings, such as larger plagiopatagium and dactylopatagium, that explain an increase in wing loading and that has negative consequences on flight efficiency and manoeuvrability of females during the reproductive period, but no differences were found related to the size of the body or the wings (de Camargo and de Oliveira, 2012).

A second study compared the efficiency of traditional morphometrics and geometric morphometrics to detect interspecific shape variation and found that geometric morphometrics improved species discrimination (Schmieder et al., 2015). A third study found sexual dimorphism in the common noctule (*N. noctula*) related to their body and wing size but failed to show any shape differences in migratory females and non-migratory males (O'Mara et al., 2016).

Here, I apply a traditional morphometric analysis of multivariate data from wings and use a geometric morphometric approach to discriminate migratory versus non-migratory populations of *L. yerbabuenae* based on changes in wing shape. As seen in chapter 2, there is no clear population structure and matrilineal gene flow is maintained between migratory and resident populations of *L. yerbabuenae;* however, each group inhabits different ecosystems and the species exhibits partial migration (Fig 2.1, chapter 2). And although it has been proposed that migratory females overwinter with non-migratory females (Galindo et al., 2004) the two populations breed allopatrically (Ceballos et al., 1997) (Rojaz-Martinez et al., 1999). I therefore presume that the thorough examination of wing parameters and the analysis of wing shape will be useful to detect subtle variation that adheres to the assumptions proposed by aerodynamic theory and flight efficiency and will be useful to differentiate individuals from the migratory and the non-migratory populations.

3.2 Objectives

The objectives of this study include the following: 1) identify morphological traits associated with migratory behaviour and foraging strategy in *L. yerbabuenae*; 2) discriminate populations based on wing size and shape using geometric morphometrics; 3) analyse the relationships among wing shape, flight performance and migratory behaviour in *L. yerbabuenae,* and 4) use the morphological data to predict flight performance of bats.

3.3 Hypotheses

I hypothesise that the wing morphology of *L. yerbabuenae* will be associated with its migratory behaviour and foraging strategy. I predict that wing shape will differ along with size among populations as variable selection pressures act upon individuals adapting them to

different ecosystems. I predict that migratory individuals will have a more efficient wing shape as an adaptation to long-distance travel and to reduce energy expenditure with higher aspect ratio, higher wing loading and more acute wing tips when compared to non-migratory females and non-migratory males; non-migratory females and males will present a shape well adapted to manoeuvring in cluttered environments such as having shorter wingspan, lower aspect ratio, lower wing loading and more rounded tips when compared to migratory individuals. Furthermore, I predict that both groups of females will have higher wing loading as a result of larger wing areas than males and explained by the effects of the additional weight that pregnant and nursing females have to carry.

3.4 Material and methods

3.4.1 Pilot study and sampling sites

I conducted a pilot study with data collected in November 2015 from bats occupying cave Los Laguitos, in Chiapas (southern Mexico) (Fig 2.1) and from bats occupying cave El Pinacate, Sonora (northern Mexico) (Fig 2.1) in May 2016. The first site corresponds to a roost of non-migratory females and the latter to a roost of migratory females. In this pilot study, I recorded the wing data using the camera of an iPhone 6 (Apple^{TM}) and a plastic grid. The results were very promising, but the methodology required improvement to provide more standardised data. Thus, I re-visited Los Laguitos in November 2016 and El Pinacate in May 2017 and added a third sampling site in Colima (central Mexico) in June 2017 where presumed non-migratory males were roosting during the summer (Fig. 2.1). At each site, I was able to capture and photograph over 150 bats and record their morphometric measurements in a more standardised way, described as follows.

3.4.2 Bat capture and handling

Bat capture methods and handling followed the same methodology as described in chapter 2. Only lactating females and males that were not reproductively active were used in this study (Mitchell-Jones and McLeish, 2004). Collection permits in accordance with The Mexican Secretariat of Environment and Natural Resources (SEMARNAT) for populations at risk or in critical habitat, permit number SGPA/DGVS/03946/15.

3.4.3 Photography

Photographs of the right wings were taken using a Sigma 105 mm F2.8 EX DG OS HSM macro lens for Nikon DSLR camera on a Nikon D750 digital SLR camera and mounted on Vanguard Alta Pro 264AB 100 aluminium tripod at 180° from the ground. The inclination of the camera and the working station were calculated using the Compass App of an iPhone 7 (Apple™) to make sure that they were perfectly parallel (0° inclination) with each other.

The camera was triggered with a remote shutter release cable to avoid moving the equipment and thus altering the focus or framing of the photographs. I used a Sigma EM-140 DG macro ring flash to improve the lighting from above and a 23 x 30 cm lightbox tracer pad below to create a contrasting image of the wing membrane, bone joints and veins. The working station was positioned underneath the camera equipment and consisted of a folding step stool with the lightbox (attached to an external battery) and a 22 x 30 cm translucent self-healing cutting mat with a grid on top (Figure 3.2).

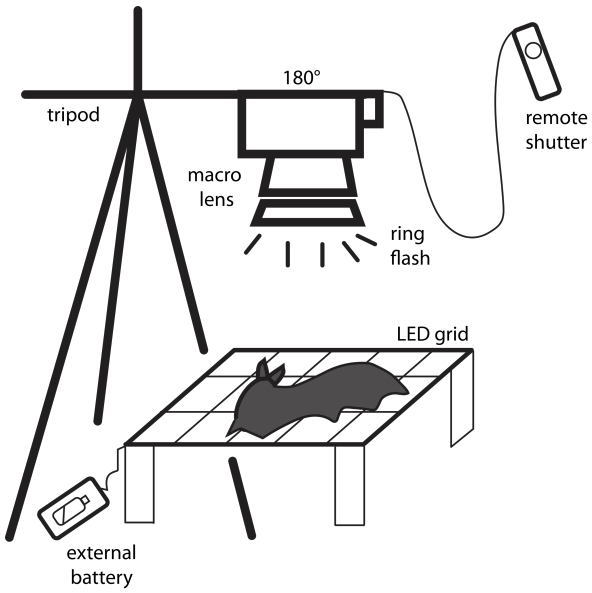


Figure 3.2 Diagram of the working station to photograph bat wings with DSLR camera mounted on a tripod at 180°, macro lens and ring flash, triggered with a remote shutter; and table with LED grid connected to an external battery. Not to scale.

To photograph the wings, I held the bat gently but firmly on the working station and exposed the right wing. An assistant then pushed the forearm to the surface and kept it as flat as possible, which in turn made all phalanges extend. I captured three photographs of each wing, opening and closing each time to reduce measurement error as the bones and wing membrane are rather flexible, and the bats would sometimes change position (Figure 3.3, Video available at goo.gl/dWKJDL); the average measurements were used in the statistical analysis.

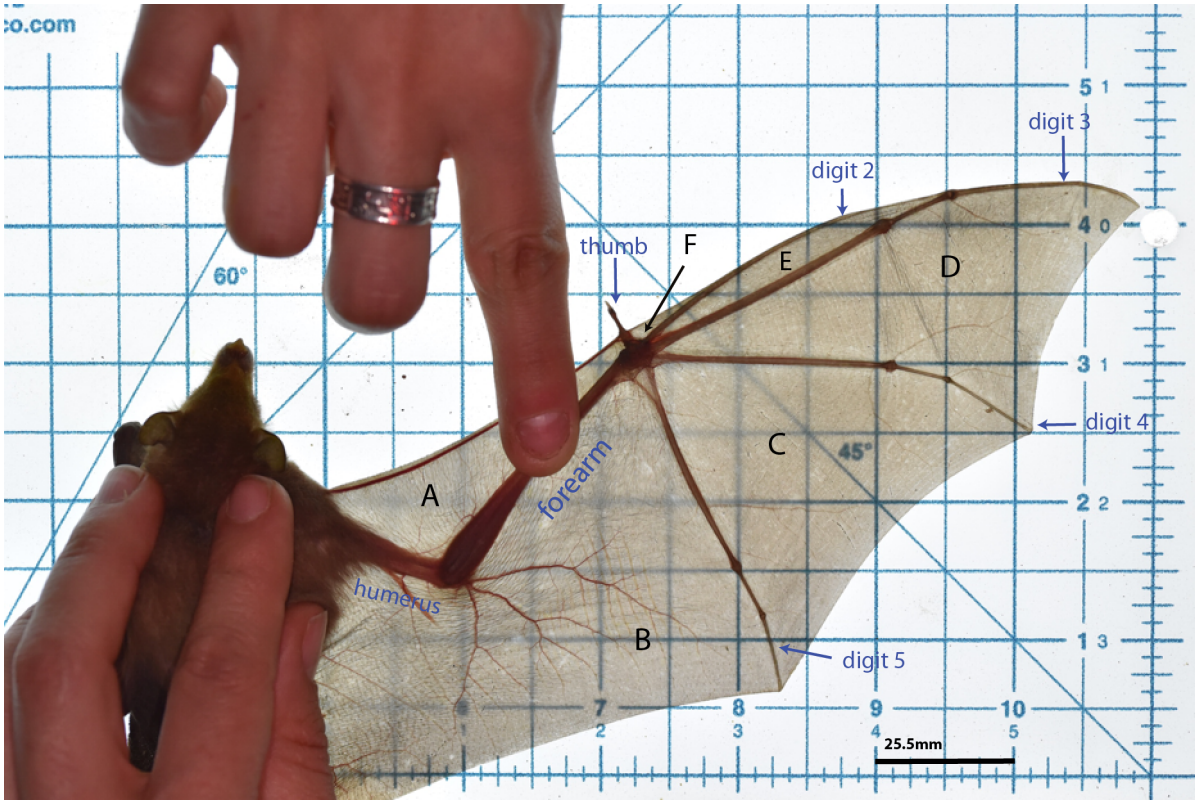


Figure 3.3 The right wing of a *L. yerbabuenae* is exposed and placed on a lit grid with all phalanges extended. The patagia are labelled as A) propatagium; B) plagiopatagium; C) dactylopatagium major; D) dactylopatagium medius; E) dactylopatagium minus; and F) dactylopatagium brevis. In blue, the humerus, forearm and the five digits.

3.4.4 Linear measurements and landmark collection

Linear measurements for some bats were taken in the field by measuring the total body length and forearm length using a digital calliper with an accuracy of 0.03 mm (iGaging IP54, USA). The body mass was measured by weighing the bats using a digital scale with a readability of 0.1 g to 1 kg and an accuracy of ± 0.1g (Asher, USA). An assessment of the reproductive status was performed on each bat (Mitchell-Jones and McLeish, 2004).

In the lab, I used ImageJ v1.44o (Rueden et al., 2017) to mine the data from photographs. The images were selected based on their quality and where all three replicates were reliable (partial wing images or blurry images were discarded). Some of the individuals photographed are the same as the ones measured directly in the field, but more bats were photographed

than those measured directly as it was faster to process them before being released. I used the forearm measurements taken with the calliper to validate the measurements from the photographs. The measurements of 13 wing bones calculated from the photographs, the total body length (measured with the calliper) and the body mass were used in the statistical analyses.

The images were calibrated with the background grid and a total of 13 linear measurements, corresponding to 13 bones (Table 3.1; Figure 3.4), were recorded using the straight-line tool. The measurements correspond to the distance between joints, which were easily recognisable by zooming into the image and always starting at the centre of the joint. For the geometric morphometric analysis, I digitised 15 landmarks with the multi-point tool, which correspond to points between the joints and the tips of the digits (Figure 3.4).

A second observer was trained to mine part of the data. To test the reliability between observations, we measured 45 bats (n=15 each group, with 3 replicates each) independently and ran the statistical analysis for the linear measurements and the landmarks. Furthermore, I performed a Spearman test of 15 landmarks and 13 linear measurements (wing bones) of the 45 individuals to investigate the correlation between observers; the results of the test show a very high correlation between the two observers for all variables ($r > 0.8$, p-values < 0.05), hence the results of both data sets were considered unbiased.

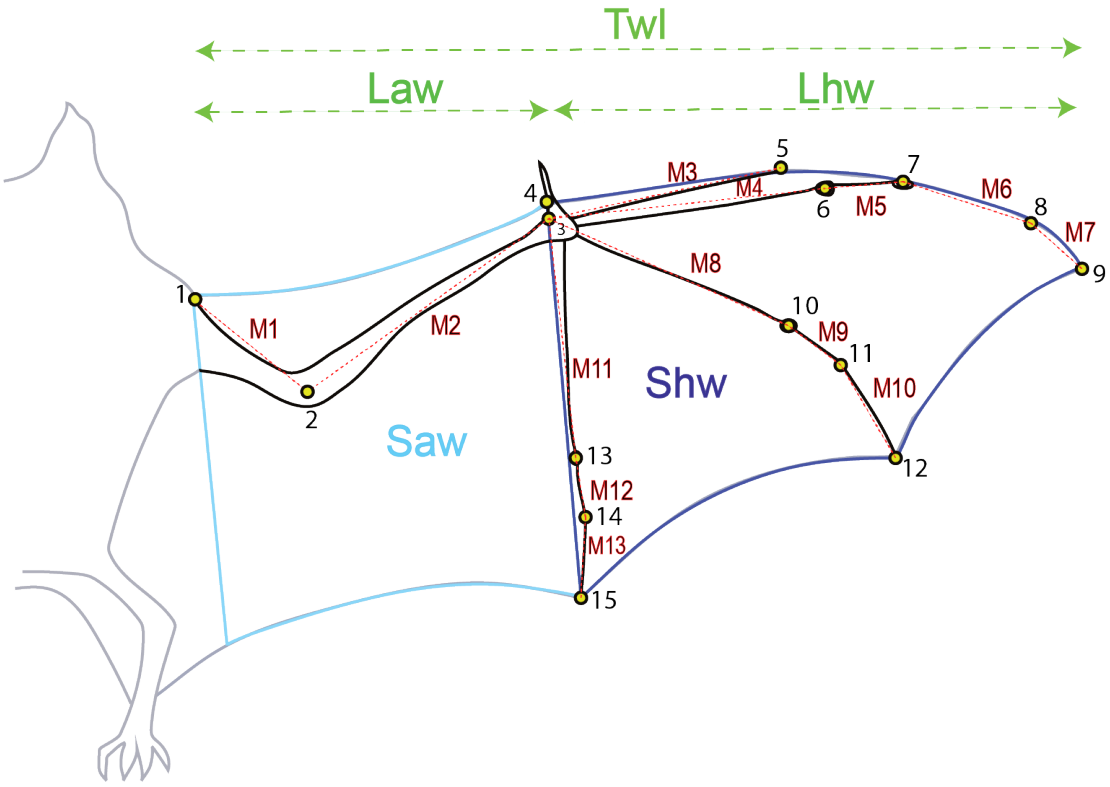


Figure 3.4 Morphological quantities to describe the wings of *L. yerbabuenae*. The red dashed lines represent 13 linear measurements of wing bones (M1-M13). The green dashed lines represent the length arm-wing (law) and the length hand-wing (lhw), the total wing length (twl) is calculated as twl = Law + lhw. In light blue, the arm-wing area (saw), and dark blue, the hand wing area (shw). The yellow dots represent 15 landmarks used to calculate changes in wing shape with geometric morphometrics.

Table 3.1 List of the 13 linear measurements (M1-M13) used to describe the wing morphology of *L. yerbabuenae* and the structure it corresponds to.

Data	Structure
M1	Humerus (mm)
M2	Forearm (mm)
M3	Metacarpal 2 (mm)
M4	Metacarpal 3 (mm)
M5	Proximal phalanx digit 3 (mm)
M6	Medial phalanx digit 3 (mm)
M7	Distal phalanx digit 3 (mm)
M8	Metacarpal 4 (mm)
M9	Proximal phalanx digit 4 (mm)
M10	Distal phalanx digit 4 (mm)
M11	Metacarpal 5 (mm)
M12	Proximal phalanx digit 5 (mm)
M13	Distal phalanx digit 5 (mm)

3.4.5 Analysis of variation of wing shape and size using traditional morphometrics

All statistical analyses were conducted in R version 3.4.1 (R Core Team, 2014). The consistency of the measurements taken with the calliper and in ImageJ was tested by comparing the means of the measurements and with a Pearson correlation as implemented in R. The average of the three linear measurements taken for each was used in the statistical analysis. The raw data were initially explored to check for normality and identify outliers with a Shapiro-Wilk normality test implemented in R.

3.4.5.1 Size correction

Morphological traits typically scale with an organism's overall body size. As a consequence, differences in trait values among individuals, within and between populations and species, will often arise simply because individuals or populations differ in body size. Although differences in size might be interesting to look at, the difference in traits becomes clearer once size has been partialled out (Berner, 2011). Furthermore, size correction is useful to study phenotypic plasticity in response to experimental treatments, or adaptive genetic divergence between populations occupying distinct habitats (Berner, 2011).

Size correction was necessary in this analysis since all three groups differ in body size significantly (Supplementary Table 3.9). Without such consideration, any differences in wing shape could be interpreted as differences in overall size and not in shape. To control for overall body size differences, I corrected the morphometric following a shearing approach (Humphries et al., 1981). Shearing is a type of residuals analysis that uses a multivariate description of "size" by pooling all measurements and analysing them with a principal component analysis (PCA); the first principal component (PC1) represents the common

allometry shared among groups, the data are then regressed against this “size” measure and the residuals compared via one-way ANOVA (McCoy et al., 2006).

3.4.5.2 Morphometric Analyses

To roughly see if the variables associated with each other I calculated Spearman’s correlations between all pairwise combinations, for each population and globally, using the package PerformanceAnalytics v1.5.2 (Peterson et al., 2015) implemented in R. Then, I used a Principal Component Analysis (PCA) to explain the variance of the dataset using packages FactoMineR v1.39 (Lê et al., 2008) and factoextra v1.0.5 (Kassambara and Mundt, 2017) in R.

To investigate further the differences among populations I compared the sample variances and sample means of the corrected linear measurements relative to each other using a one-way ANOVA with an Honestly Significantly Different Tukey post-hoc test (TukeyHSD) as implemented in R. The models were checked for normality with a Shapiro-Wilk’s W test, a Kolmogorov–Smirnov test (KS-test) and validated by visual examination of residuals plots following the guidelines of Zuur et al. (2010); if the data did not conform to normality, I used a log transformation to reduce skewness before the TukeyHSD test.

3.4.6 Measurement of morphological parameters

The parameters used to describe the wings of *L. yerbabuenae* were defined following Norberg and Rayner (1987):

The wingspan (B) is the distance between the two wingtips, including the body width. However, I did not include the body width since I was not able to record this measurement

reliably from the photographs. Thus, the wingspan (B) was calculated as 2x the total wing length (twl), which is the length of the wing distal to the humeral joint. Other authors have used a similar approach to calculate the wingspan (e.g. Struhsaker 1961; Hartman 1963; de Camargo & de Oliveira 2012; O'Mara et al. 2016).

The hand-wing area (shw) was calculated by defining a polygon from the thumb to the tip of the third digit and the tip of the fifth digit, including the dactylopatagium major, the dactylopatagium medium, and the dactylopatagium minus (Figure 3.3). A bordering polygon defined the arm-wing area (saw) from the thumb to the tip of the fifth digit, the border of the plagiopatagium, the humeral joint and the propatagium. To consistently record the arm-wing area, without the positioning of the foot altering the measurement, the angle from the thumb to the tip of the fifth digit was recorded and used to calculate the angle from the humeral joint and the border of the plagiopatagium (Figure 3.4). The total wing area (S) was calculated as the sum of the hand-wing area (shw) and the arm-wing area (saw).

The aspect ratio (A) was calculated as the square of the wingspan divided by the wing area (S). $A=B^2/S$. The wing loading (Nm^{-2}) was calculated as the body mass (M) multiplied by the gravitational force (g) divided by the wing area (S) multiplied by the body mass and elevated at the power of 1/3 to control for body body size effects (M*g/S*M^(1/3). Since wing area increases more slowly with body size than body mass, bigger bats inherently have higher wing loadings; this scaling compensates for body size effects to produce a relative measurement of wing loading (Norberg, 1994). The tip length ratio (Ti), which is associated with manoeuvrability, was calculated as the ratio of the length of the hand-wing (lhw) to the length of the arm-wing (law); Ti = lhw/law (Figure 3.4). The tip area ratio (Ts) was calculated as the

ratio of the hand-wing area (shw) to the arm-wing area (saw); Ts = shw/saw. The tip shape index (I) was calculated as I = Ts/(Ti-Ts) and is valuable in describing the wingtip shape independent of the extent of the hand-wing (Norberg and Rayner, 1987). A summary of these equations can be found in Table 3.2.

Table 3.2 Summary of parameters for the description of bat wings as proposed by Norberg & Rayner (1994)

Wing parameter	Acronym	Equation
length arm-wing (mm)	law	
length hand-wing (mm)	lhw	
hand-wing area (mm^2)	shw	
arm-wing area (mm^2)	saw	
total wing length (mm)	twl	twl = law + lhw
total wing area ($mm^{2)}$	S	shw + saw
wingspan (mm)	B	2x(twl)
aspect ratio	A	$A = B^2/S$
wing loading	Nm^{-2}	M*g/S*M^(1/3)
tip length ratio	Ti	Ti = lhw/law
tip area ratio	Ts	Ts = shw/saw
tip shape index	I	I = Ts/(Ti-Ts)

3.4.7 Analysis of variation of wing shape using geometric morphometrics

I estimated shape using geometric morphometric techniques from 15 landmark coordinates. The landmarks were acquired using the multi-point tool in ImageJ (Rueden et al., 2017) and correspond to easily recognisable structures of the wings such as the humeral joint, the forearm joint, four metacarpal joints, the medial, proximal and distal interphalangeal joints of digits 2-5, and the tips of the wing (Figure 3.4). I digitised the landmarks of 300 bats (n=100 for each population, with 3 replicates for each). The averaged landmarks were used to identify partial warps (variations in wing shape) by the Generalized Procrustes Analysis (GPA), where the landmarks are rotated, translated, and scaled to remove the effects of size and other non-shape parameters using MorphoJ v1.06d (Klingenberg, 2011). The superimposed dataset was then used to describe patterns of variation in shape with a Principal Component Analysis

(PCA) and a subsequent Canonical Variate Analysis (CVA), which determines if pre-defined groups can be statistically differentiated based on multivariate data (Leandro et al., 2016) (Figure 3.5). I performed a multivariate regression of Procrustes coordinates against centroid size to account for the potential effect of size in shape. I measured Mahalanobis distances of the statistical significance of pairwise differences in mean shapes with 10,000 permutations to measure how similar each population was to another; smaller values of Mahalanobis distances (MD) between two groups means they are more closely related to each other (Klingenberg, 2016; Leandro et al., 2016)

3.4.8 Flight power and speed

I predicted the flight speed of the bats in each population by calculating the minimum power flight speed (Vmp) at which bats should fly to reduce power expenditure (maximum endurance) and maximum range flight speed (Vmr) at which bats achieve maximum flight range for a given amount of energy (Oleksy et al., 2015; Holderied and Jones, 2003; Pennycuick, 2008) by using Flight v 1.25 as described in Pennycuick's model (Pennycuick, 2008). The program calculates a mechanical power curve (the rate at which flight muscles do mechanical work in steady flight) and a chemical curve (the rate at which chemical fuel energy is consumed) in relation to flight speed, and is derived from body mass, wing span, wing area and aspect ratio (Holderied and Jones, 2003). The power curves were estimated by averaging the wing data of each population and assuming bats would fly at sea level with an air density of 1.23 kg/cubic m (Holderied and Jones, 2003; Pennycuick, 2008).

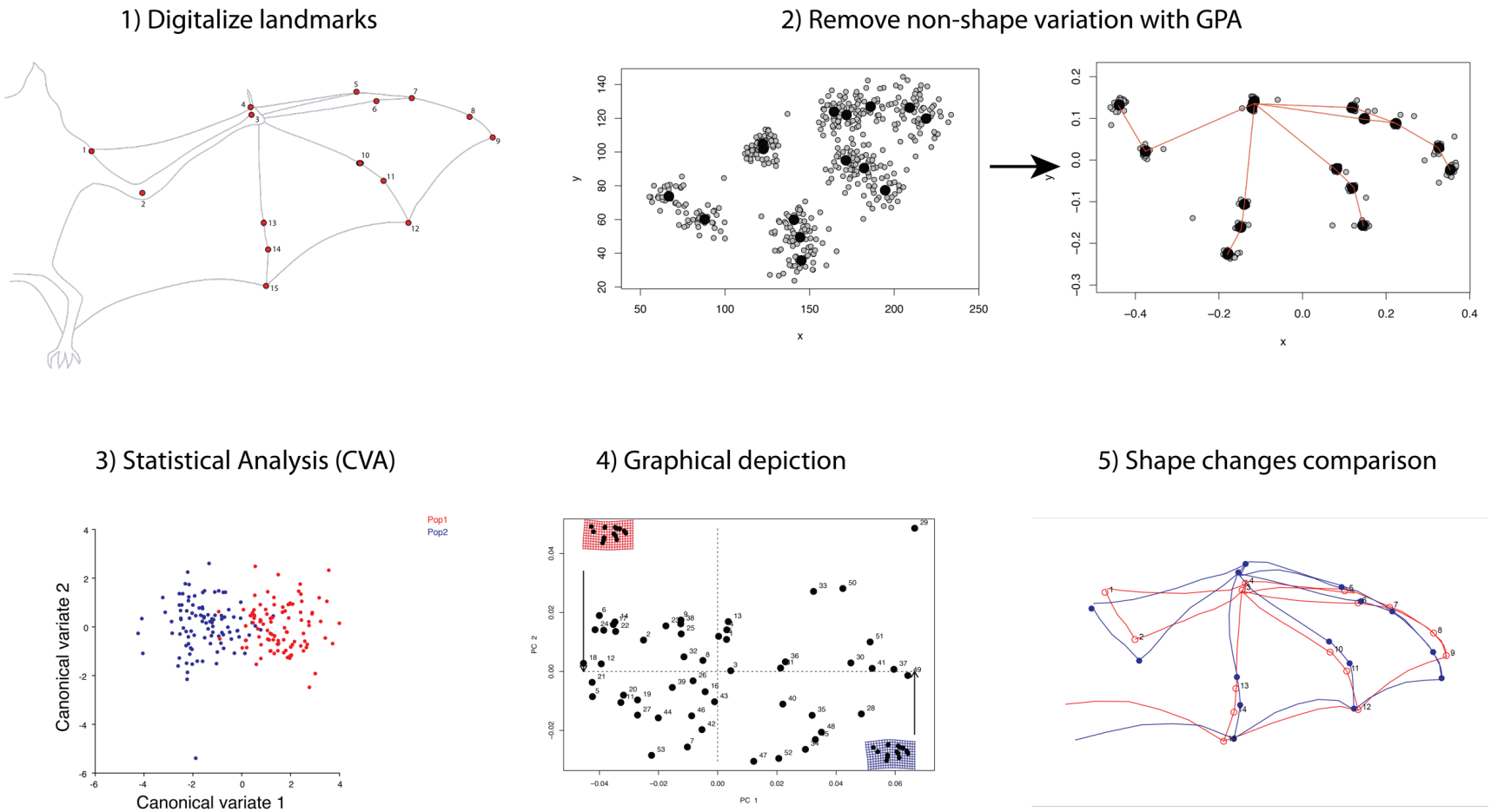


Figure 3.5 Steps of the Procrustes paradigm for landmark-based geometric morphometrics. 1) digitalise raw data; 2) Generalized Procrustes Analysis (GPA) to remove non-shape variation; 3) statistical analysis with Canonical Variance Analysis (CVA); 4) graphical depiction of results; 5) shape change comparison.

3.5 Results

Traditional Morphometrics

3.5.0 Size correction

The exploratory PCA (Figure 3.6) of the raw variables shows that the loadings of PC1 range from 1.41 to 10.77 (Table 3.3), indicating that PC1 is an allometric vector describing correlated changes in both size and shape, if instead PC1 were a pure scaling (size) isometric vector, the loadings of all variables on this axis would be 3.61, calculated as $1/\sqrt{13}$ (Jolicoeur, 1963; Sidlauskas, et al. 2011). As PC1 represents allometric variation, raw separation amongst groups on this axis reflects differences in the size or age/sex structure of the various samples and necessitates regression to test for amongst-group differences. To correct for this variation in size, the raw variables were regressed against PC1 and the residuals were used in the morphometric analysis; from here on the residuals are referred as the "corrected variables".

Table 3.3 Variable loadings explained for the first five principal component axes of an exploratory PCA for 13 raw morphometric measurements (M1-M13) of 300 specimens of *L. yerbabuenae* from three populations (Male, Migratory, Resident).

	PC1	*PC2*	*PC3*	*PC4*	*PC5*
M1	1.41	27.96	29.48	19.00	18.74
M2	8.32	6.84	0.73	0.00	0.16
M3	8.92	0.94	6.00	4.75	0.34
M4	10.60	0.70	8.30	3.37	1.06
M5	6.98	8.03	8.00	2.86	14.34
M6	10.13	2.31	0.04	0.25	5.47
M7	1.77	25.79	7.80	49.30	12.81
M8	10.77	1.50	7.78	2.38	0.56
M9	8.88	0.94	10.67	3.42	5.72
M10	8.20	12.12	1.77	2.05	6.51
M11	10.23	2.85	2.96	2.97	0.32
M12	8.03	0.62	6.88	4.75	8.15
M13	5.76	9.40	9.59	4.90	25.82

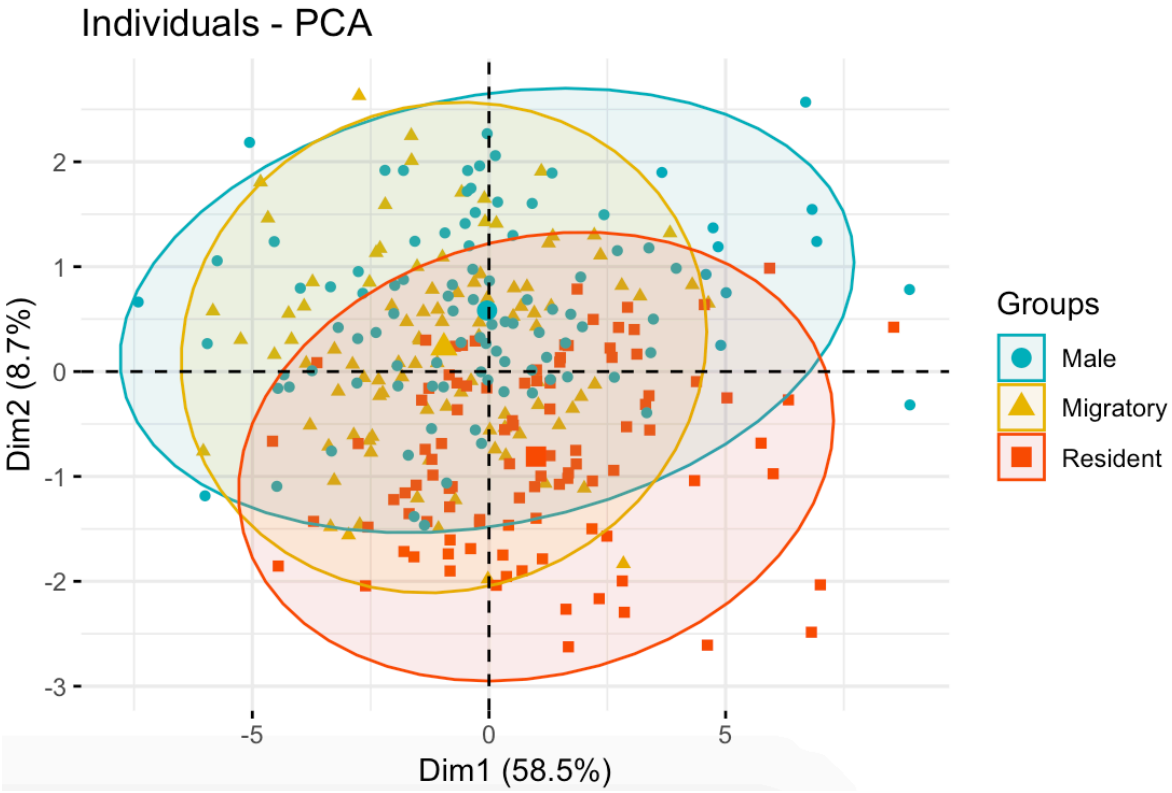


Figure 3.6 Scatterplot of principal components one and two from PCA of 13 raw linear measurements (M1-M13) of *L. yerbabuenae* from three populations (Male, Migratory and Resident); ellipses represent 95% confidence intervals. Populations are colour coded: in green: males, purple: migratory, red: resident. Larger icons represent the centre of the ellipse.

3.5.1 Tests for normality

The Shapiro-Wilk normality test of the corrected measures showed that some variables did not conform to normality ($p<0.05$); including M1 and M13 in males, M1, M7 and M13 in the migratory population and M10 in the resident population (Table 3.4). These variables were log-transformed to approximate a standard normal distribution.

Table 3.4 Shapiro-Wilk Normality test of the corrected data of 13 corrected linear measurements (M1-M13). Significance: "0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' '

	Males		*Migratory*		*Resident*	
	W	p-value	W	p-value	W	p-value
M1	0.94	0.00	0.92	0.00	0.99	0.57
M2	0.99	0.75	0.99	0.72	0.98	0.16
M3	0.99	0.71	0.99	0.44	0.99	0.49
M4	0.99	0.32	0.99	0.87	0.98	0.13
M5	0.99	0.32	0.99	0.46	0.99	0.68
M6	0.99	0.67	0.97	0.05	0.99	0.89
M7	0.99	0.91	0.97	0.03	0.98	0.31
M8	0.99	0.75	0.98	0.23	0.99	0.36
M9	0.99	0.51	0.99	0.78	0.98	0.27
M10	0.99	0.78	0.99	0.51	0.96	0.01
M11	0.98	0.29	0.99	0.92	0.99	0.83
M12	0.99	0.73	0.98	0.28	0.99	0.74
M13	0.68	0.00	0.97	0.01	0.99	0.32

3.5.2 Correlation matrix of 13 linear morphometric measurements

Spearman's correlation matrices of pairwise comparisons of the 13 corrected linear morphometric measurements (Figure 3.7) show a strong positive association ($r>0.5$, $p<0.001$) between the length of the metacarpals of the third (M4) and fourth fingers (M8) in all three groups (Migratory, Resident, Males).

The variables with the strongest positive correlations ($r>0.5$, $p<0.001$) for migratory females (Figure 3.7) occur between metacarpals 3 and 4 (M4-M8); moderate correlations ($r<0.5$, >0.3, $p<0.001$) occur between metacarpals 4 and 5 (M8-M11), metacarpals 2 and 4 (M3-M8), the medial phalanx of digit 3 and the distal phalanx of digit 4 (M6-M10), the proximal phalanx digit 3 and the proximal phalanx digit 4 (M5-M9), the proximal phalanx digit 4 and the proximal phalanx digit 5 (M9-M12), and the distal phalanx digit 4 and the distal phalanx digit 5 (M10-M13).

For resident females (Figure 3.7) the strongest positive correlations ($r>0.5$, $p<0.001$) also occur between metacarpals 3 and 4 (M4-M8). Moderate positive correlations ($r <0.5, >0.3$, $p<0.001$) occur between metacarpals 4 and 5 (M8-M11), the proximal phalanx of digit 4 and the proximal phalanx of digit 5 (M9-M12), and metacarpals 2 and 3 (M3-M4), the proximal phalanx digit 3 and the proximal phalanx digit 5 (M5-M12), the distal phalanx digit 4 and the distal phalanx digit 5 (M10-m13)

For non-migratory males (Figure 3.7) the strongest positive correlations ($r>0.5$, $p<0.001$) also occur between metacarpals 3 and 4 (M4-M8), while moderate correlations ($r <0.5, >0.3$, $p<0.001$) ocurr between (M3-M4), (M2-M11), the proximal phalanx digit 3 and the proximal phalanx digit 4 (M5-M9), the medial phalanx of digit 3 and the distal phalanx of digit (M6-M10), metacarpals 4 and 5 (M8-M11).

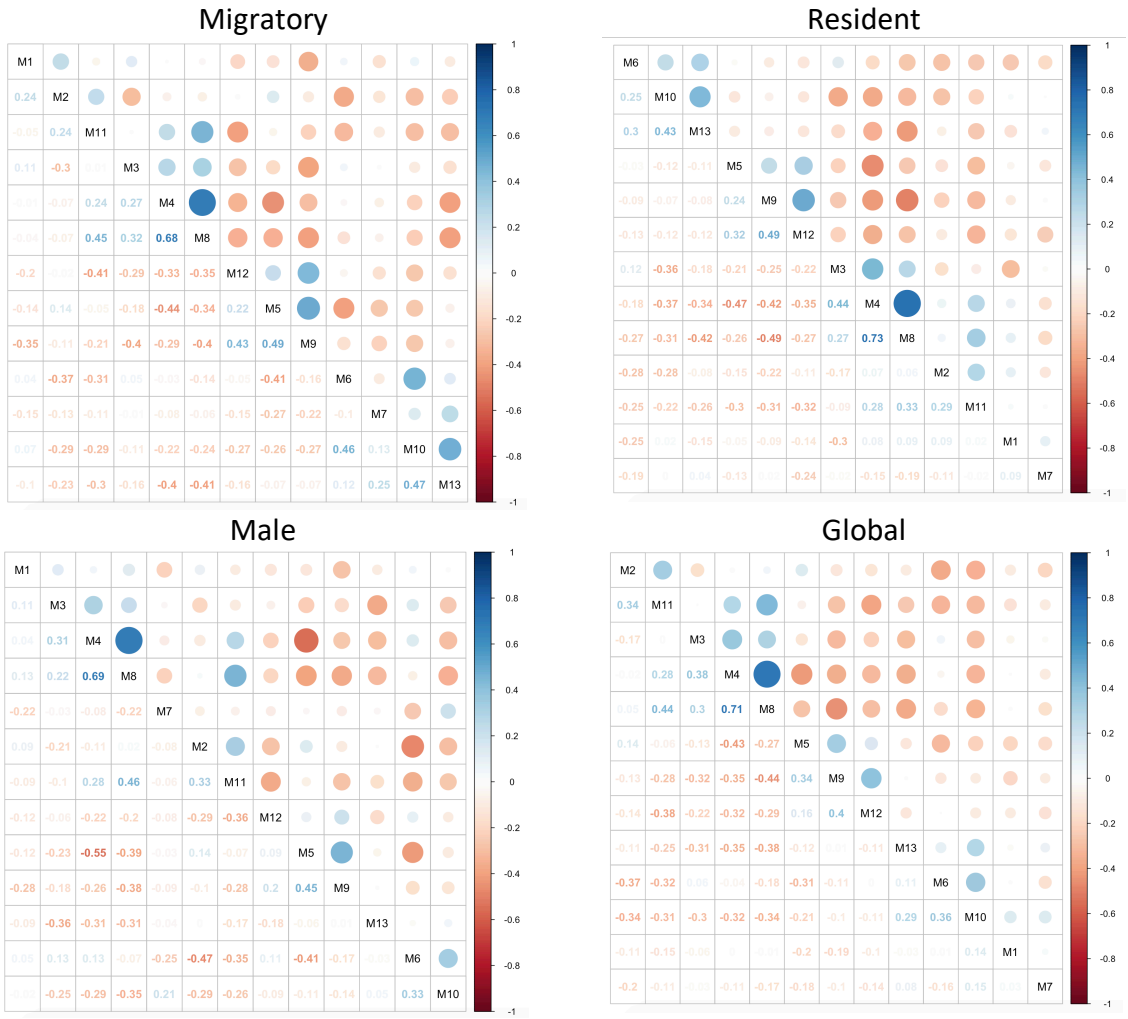


Figure 3.7 Correlation matrices of 13 corrected linear measurements (M1-M13) of 100 individuals of *L. yerbabuenae* from each of the three populations (Male, Migratory, and Resident) and correlations in all three populations (n=300) combined (Global). On the bottom of the diagonal: the correlation coefficients (r) are displayed; on the top of the diagonal: positive correlations are displayed in blue and negative correlations in red. Colour intensity and the size of the circle are proportional to the correlation coefficients.

3.5.3 Principal Component Analysis (PCA)

The PCA of the 13 corrected linear morphometric measurements extracted two components (eigenvalue > 1), which accounted for 42 % of the cumulative variance in the dataset (Table 3.6). Size variables in the PCA had component loadings ranging from -0.56 to 0.85 (Table 3.5)(Figure 3.9). The scatterplot of principal components (Figure 3.8) revealed that the first two components (PC1 and PC2) explained 42 % of the variance. For PC1, the characters with

the highest loadings (contribution % >7) were metacarpal 4 (M8), metacarpal 3 (M4), metacarpal 5 (M11), the proximal phalanx digit 4 (M9), and metacarpal 2 (M3).

For PC2 the characters with the highest loadings (contribution % >7) were the proximal phalanx digit 3 (M5), the medial phalanx digit 3 (M6), the distal phalanx digit 4 (M10), the troximal phalanx digit 5 (M12), and the proximal phalanx digit 4 (M9). The combined contributions of PC1 and PC2 can be seen as a bar plot in Figure 3.9.

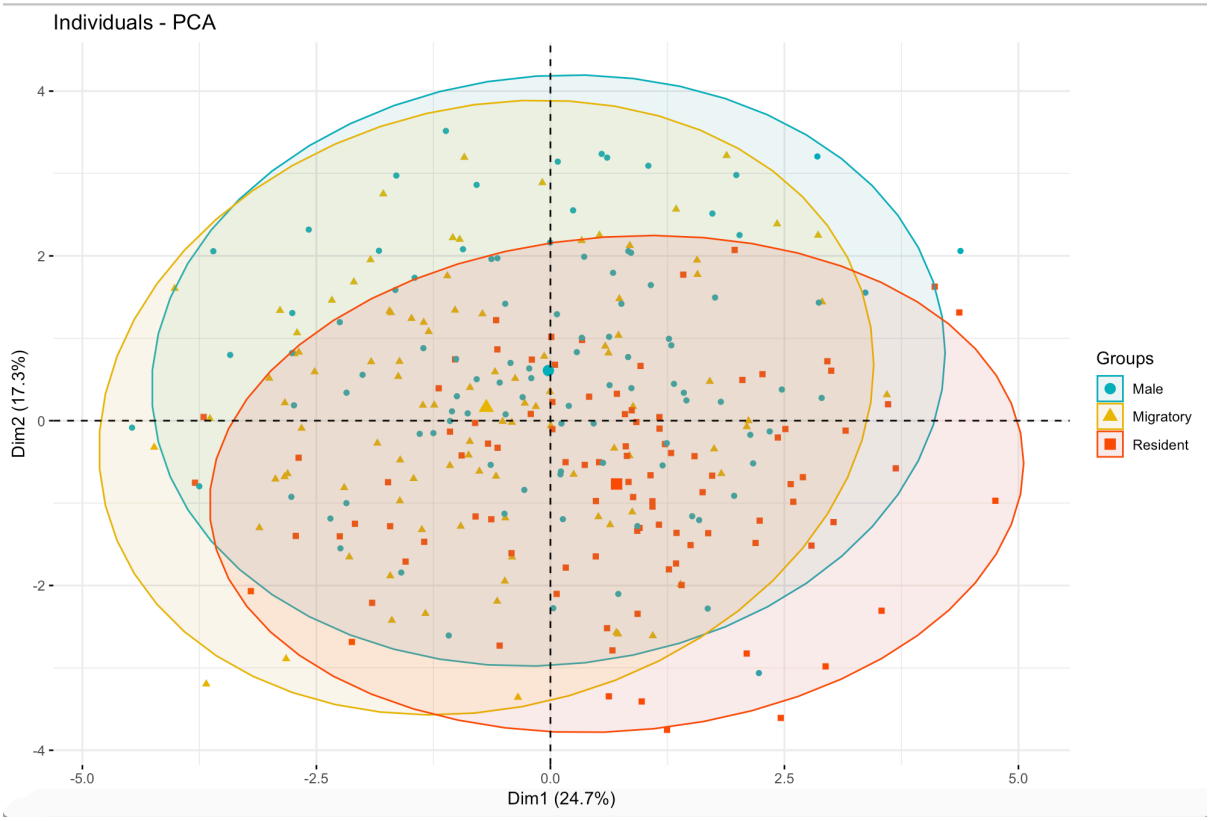


Figure 3.8 Scatterplot of principal components one and two from PCA of 13 corrected linear measurements (M1-M13) of *L. yerbabuenae* from three populations (Male, Migratory and Resident); ellipses represent 95% confidence intervals. Populations are colour coded: in green: males, purple: migratory, red: resident. Larger icons represent the centre of the ellipse.

Table 3.5 Variable loadings explained for the first five principal component axes of a PCA for 13 corrected morphometric measurements (M1-M13) of 300 specimens of *L. yerbabuenae* from three populations (Male, Migratory, Resident).

	PC1	*PC2*	*PC3*	*PC4*	*PC5*
M1	0.00	0.35	-0.11	0.13	0.86
M2	0.25	-0.54	-0.48	-0.27	0.11
M3	0.50	0.18	0.46	0.24	-0.26
M4	0.81	0.19	0.23	-0.01	0.01
M5	-0.30	-0.69	0.04	0.07	-0.06
M6	-0.22	0.61	0.34	-0.47	-0.16
M7	-0.12	0.28	-0.30	0.82	-0.17
M8	0.85	0.05	0.11	-0.05	0.06
M9	-0.56	-0.46	0.29	0.08	-0.03
M10	-0.47	0.61	-0.24	-0.13	0.01
M11	0.63	-0.28	-0.40	-0.13	-0.09
M12	-0.47	-0.32	0.52	0.00	0.22
M13	-0.46	0.28	-0.45	-0.18	-0.27

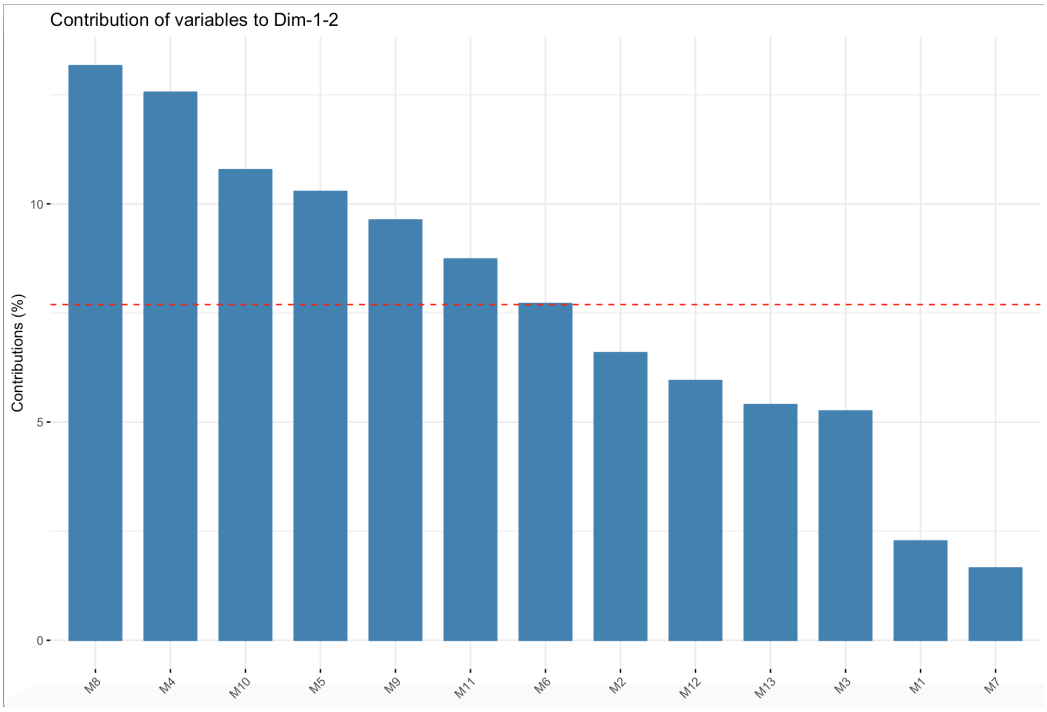


Figure 3.9 Bar plot of combined variable contributions to component 1 and 2 to the PCA of 13 corrected linear measurements (M1-M13) of *L. yerbabuenae* from three populations (Male, Migratory and Resident); the red dashed line indicates the expected average contribution.

Table 3.6 Variances of the principal components of 13 morphometric measurements for 300 specimens of *L. yerbabuenae* in three populations (Male, Migratory and Resident).

	Eigenvalue	*% of variance*	*Cumulative %*
comp 1	3.21	24.68	24.68
comp 2	2.25	17.28	41.96
comp 3	1.51	11.61	53.57
comp 4	1.12	8.59	62.16
comp 5	1.02	7.81	69.98
comp 6	0.87	6.70	76.67
comp 7	0.73	5.64	82.32
comp 8	0.68	5.20	87.51
comp 9	0.54	4.15	91.66
comp 10	0.46	3.55	95.22
comp 11	0.39	2.96	98.18
comp 12	0.22	1.70	99.88
comp 13	0.02	0.12	100.00

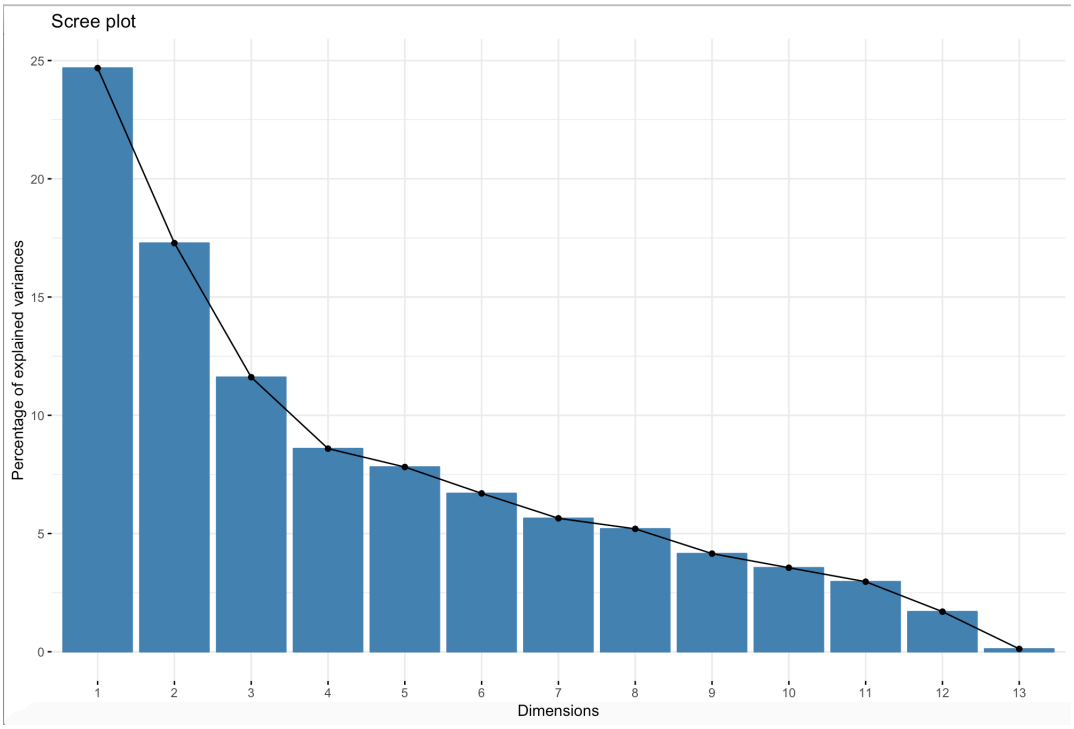


Figure 3.10 Scree plot of percent variance explained by the number of principal components (dimensions) of 13 corrected morphometric measurements for 300 specimens of *L. yerbabuenae* in three populations (Male, Migratory and Resident).

3.5.5 Variation among populations

The results of the one-way ANOVA with the TukeyHSD test show the difference of means of the 13 corrected variables among populations (Table 3.7). A comparison of the Tukey post-hoc test of the linear measurements is shown as a forest plot of the difference of means with 95% CI (Figure 3.11). The results are also shown as boxplots with levels of significance of the TukeyHSD (Figure 3.12). A list of the variation in mean values (millimetres) among populations can be found in Supplementary Table 3.10.

Significant differences among all populations were found in the length of the humerus (M1) with males having longer structures than migratory females and resident females having the shortest; inversely, differences in the length of the forearm (M2) show that resident females have the longest structures and males the shortest. The lengths of metacarpal 2 (M3) and metacarpal 3 (M4) are both of similar size in male and resident females, but shorter in migratory females.

The proximal phalanx of digit 2 (M5) is longer in resident females than in males and migratory females. The length of the medial phalanx of digit 3 (M6) is not significantly different among the three populations. The distal phalanx digit 3 (M7) is smaller in residents than in males but not significantrly different between migratory females and males or migratory females and resident females. Metacarpal 4 (M8) is larger in resident than in migratory females but not significantly different between females and males.

The length of the proximal phalanx of digit 4 (M9) is larger in migratory females and comparable between resident females and males. The distal phalanx digit 4 (M10) is shorter

in resident females and comparable in males and migratory females. Metacarpal 5 (M11) is larger in resident females and comparable between migratory females and males. The proximal phalanx digit 5 (M12) and the distal phalanx of digit 5 (M13) are larger in migratory females than in resident females but comparable with that of males.

Table 3.7 Results of the Tukey post-hoc test (TukeyHSD) of log-transformed data of 13 corrected measurements (M1-M13) of *L. yerbabuenae* from three populations (Male, Migratory and Resident). Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' '

Response	*Independent Variable*	*Difference of the means*	*Lower CI 95%*	*Upper CI 95%*	*p-value (adjusted)*
	Migratory-Male	-0.01	-0.02	0.00	0.001
	Resident-Male	-0.03	-0.04	-0.02	0.000
M1	Resident-Migratory	-0.02	-0.03	-0.01	0.000
	Migratory-Male	0.01	0.00	0.01	0.000
	Resident-Male	0.01	0.01	0.01	0.000
M2	Resident-Migratory	0.00	0.00	0.01	0.000
	Migratory-Male	-0.01	-0.01	0.00	0.000
	Resident-Male	0.00	0.00	0.00	0.663
M3	Resident-Migratory	0.01	0.00	0.01	0.000
	Migratory-Male	0.00	0.00	0.00	0.038
	Resident-Male	0.00	0.00	0.00	0.860
M4	Resident-Migratory	0.00	0.00	0.00	0.009
	Migratory-Male	0.00	-0.01	0.00	0.842
	Resident-Male	0.01	0.00	0.01	0.001
M5	Resident-Migratory	0.01	0.00	0.01	0.000
	Migratory-Male	0.00	0.00	0.01	0.580
	Resident-Male	0.00	-0.01	0.00	0.461
M6	Resident-Migratory	0.00	-0.01	0.00	0.076
	Migratory-Male	-0.01	-0.01	0.00	0.063
	Resident-Male	-0.01	-0.02	-0.01	0.000
M7	Resident-Migratory	-0.01	-0.01	0.00	0.134
	Migratory-Male	0.00	0.00	0.00	0.319
	Resident-Male	0.00	0.00	0.00	0.174
M8	Resident-Migratory	0.00	0.00	0.00	0.004
	Migratory-Male	0.00	0.00	0.01	0.010
	Resident-Male	0.00	0.00	0.00	0.958
M9	Resident-Migratory	-0.01	-0.01	0.00	0.004
	Migratory-Male	0.00	0.00	0.01	0.563
	Resident-Male	-0.01	-0.01	0.00	0.000
M10	Resident-Migratory	-0.01	-0.01	0.00	0.000
	Migratory-Male	0.00	0.00	0.00	0.898
M11	Resident-Male	0.00	0.00	0.01	0.001

	Resident-Migratory	0.00	0.00	0.01	0.000
	Migratory-Male	0.00	0.00	0.01	0.165
	Resident-Male	0.00	-0.01	0.00	0.719
M12	Resident-Migratory	0.00	-0.01	0.00	0.027
	Migratory-Male	0.01	0.00	0.01	0.052
	Resident-Male	0.00	-0.01	0.00	0.287
M13	Resident-Migratory	-0.01	-0.02	0.00	0.000

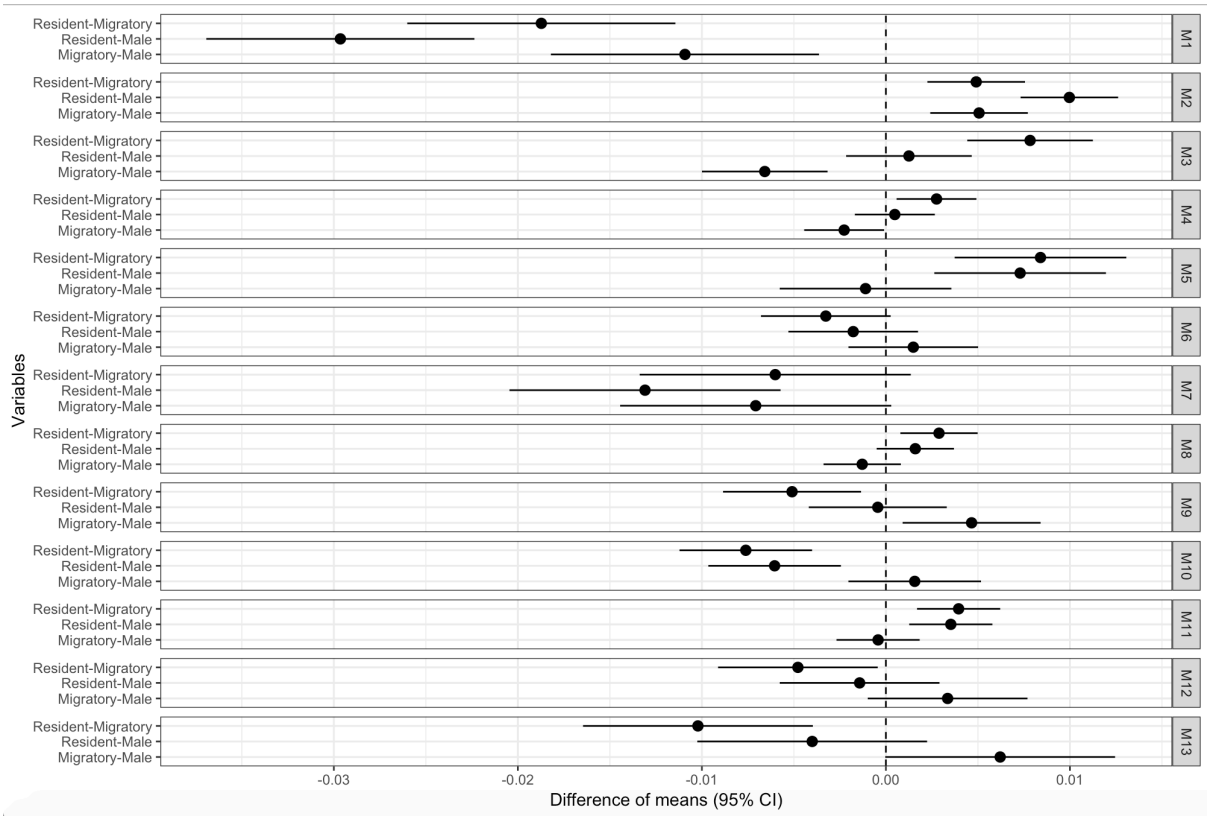


Figure 3.11 Forest plot of standardised mean differences of 13 corrected linear measurements (M1-M13) of *L. yerbabuenae* from three populations (Migratory, Resident and Male) with 95% Confidence Interval. Difference of the means (effect sizes) and 95% confidence intervals arising from the TukeyHSD test are shown with black dots and solid black lines, respectively.

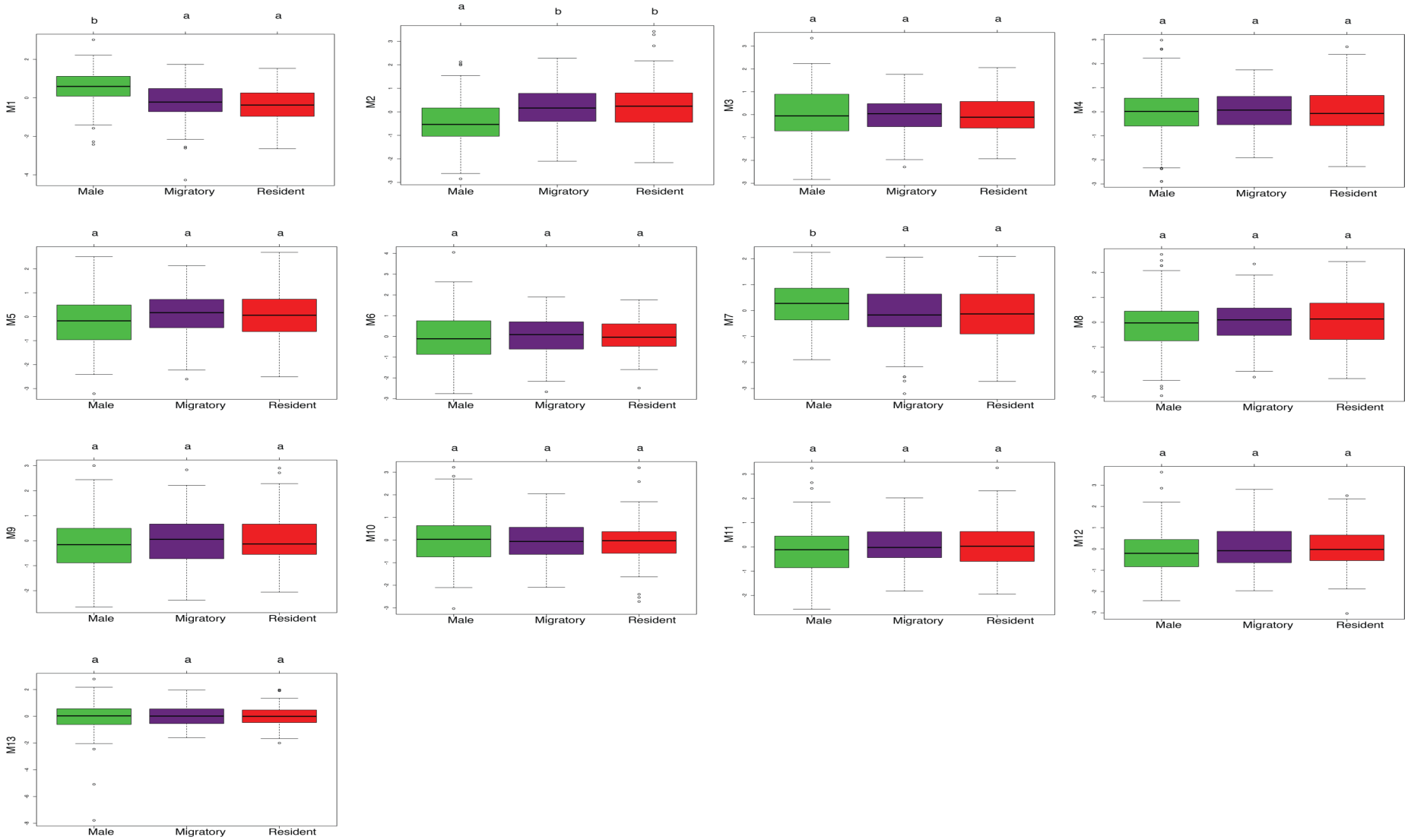


Figure 3.12 Box plots showing the variation of 13 corrected linear measurements (M1-M13) of *L. yerbabuenae* in three populations (Migratory, Resident and Male). Box plots followed by a different letter are significantly different according to TukeyHSD test at a 0.01 level of significance. The y-axis is the value of the residuals.

3.5.6 Variation in wing parameters

The results of the TukeyHSD test for the wing parameters (Table 3.8; Figure 3.14) show that the length of the hand-wing (lhw), the arm-wing area (saw), and the wingspan (B) of the migratory population are significantly smaller than in males or resident females. Similarly, the hand-wing area (shw) was significantly different in all groups, with the migratory females showing the lowest values. The tip area ratio (Ts) and the tip shape index (I) were significantly higher in resident females than in migratory females and males. The aspect ratio (A) and the wing loading (WL) were significantly higher in migratory females and comparable in males and residents. No significant differences were found among populations in the length of the arm-wing (law), the tip length ratio (Ti), or the body mass (W). Males had significantly larger bodies (b) than migratory and resident females. A comparison of the Tukey post-hoc test of the wing parameters is also shown as a forest plot of the difference of means with 95% CI (Figure 3.13). A list of the variation in mean values among populations can be found in Supplementary Table 3.10.

Table 3.8 Results of the Tukey post-hoc test (TukeyHSD) of parameters used for the description of the wings of *L. yerbabuenae* from three populations (Male, Migratory and Resident). Arm-wing length (law), hand-wing length (lhw), hand-wing area (shw), arm-wing area (saw), tip length ratio (Ti), tip area ratio (Ts), tip shape index (I), aspect ratio (A), wingspan (B), body mass (W), wing loading (WL).

Response	*Independent Variable*	*Difference of the means*	*Lower CI 95%*	*Upper CI 95%*	*p-value (adjusted)*
law	Migratory-Male	-0.717	-2.019	0.585	0.398
	Resident-Male	0.230	-1.072	1.533	0.909
	Resident-Migratory	0.947	-0.355	2.250	0.202
lhw	Migratory-Male	-2.098	-3.167	-1.029	0.000
	Resident-Male	0.432	-0.637	1.501	0.607
	Resident-Migratory	2.531	1.462	3.600	0.000
shw	Migratory-Male	-309.101	-412.484	-205.717	0.000
	Resident-Male	109.553	6.169	212.936	0.035
	Resident-Migratory	418.653	315.269	522.037	0.000
saw	Migratory-Male	-234.937	-353.650	-116.223	0.000
	Resident-Male	-70.212	-188.925	48.502	0.346
	Resident-Migratory	164.725	46.012	283.438	0.003
Ti	Migratory-Male	-0.012	-0.042	0.018	0.605
	Resident-Male	0.003	-0.027	0.033	0.978
	Resident-Migratory	0.015	-0.015	0.045	0.479
Ts	Migratory-Male	-0.007	-0.035	0.022	0.834
	Resident-Male	0.049	0.020	0.077	0.000
	Resident-Migratory	0.055	0.027	0.084	0.000
I	Migratory-Male	0.047	-0.168	0.263	0.862
	Resident-Male	0.593	0.377	0.808	0.000
	Resident-Migratory	0.545	0.330	0.760	0.000
A	Migratory-Male	0.400	0.203	0.598	0.000
	Resident-Male	0.040	-0.157	0.238	0.880
	Resident-Migratory	-0.360	-0.558	-0.163	0.000
B	Migratory-Male	-5.630	-9.693	-1.567	0.004
	Resident-Male	1.325	-2.738	5.389	0.723
	Resident-Migratory	6.956	2.893	11.019	0.000
W	Migratory-Male	0.296	-0.398	0.991	0.574
	Resident-Male	-0.112	-0.807	0.582	0.923
	Resident-Migratory	-0.408	-1.074	0.257	0.319
WL	Migratory-Male	0.000	0.000	0.000	0.000
	Resident-Male	0.000	0.000	0.000	0.719
	Resident-Migratory	0.000	0.000	0.000	0.000

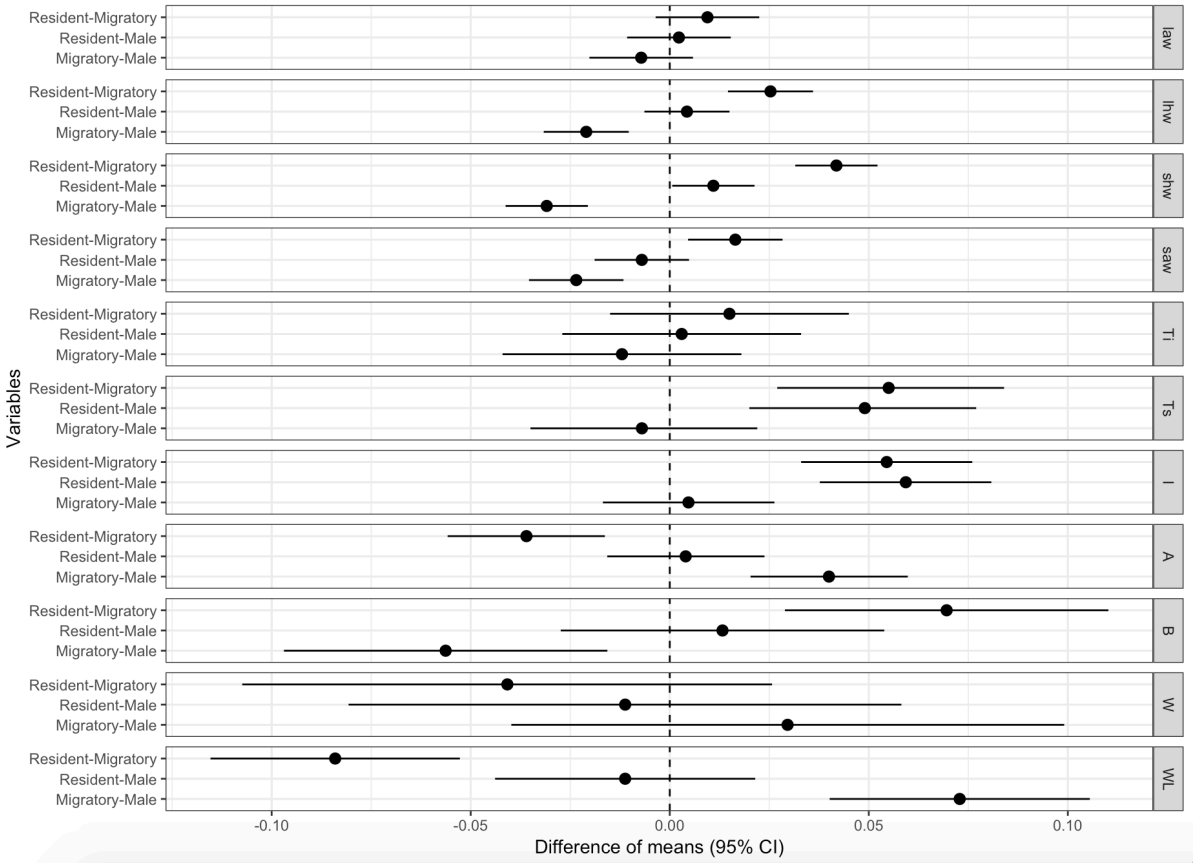


Figure 3.13 Forest plot of standardised mean differences (log transformed) of wing parameters used for the description of *L. yerbabuenae* from three populations (Migratory, Resident and Male) with 95% Confidence Interval. Difference of the means (effect sizes) and 95% confidence intervals arising from the TukeyHSD test are shown with white circles and solid black lines, respectively. Arm-wing length (law), hand-wing length (lhw), hand-wing area (shw), arm-wing area (saw), tip length ratio (Ti), tip area ratio (Ts), tip shape index (I), aspect ratio (A), wingspan (B), body mass (W), wing loading (WL).

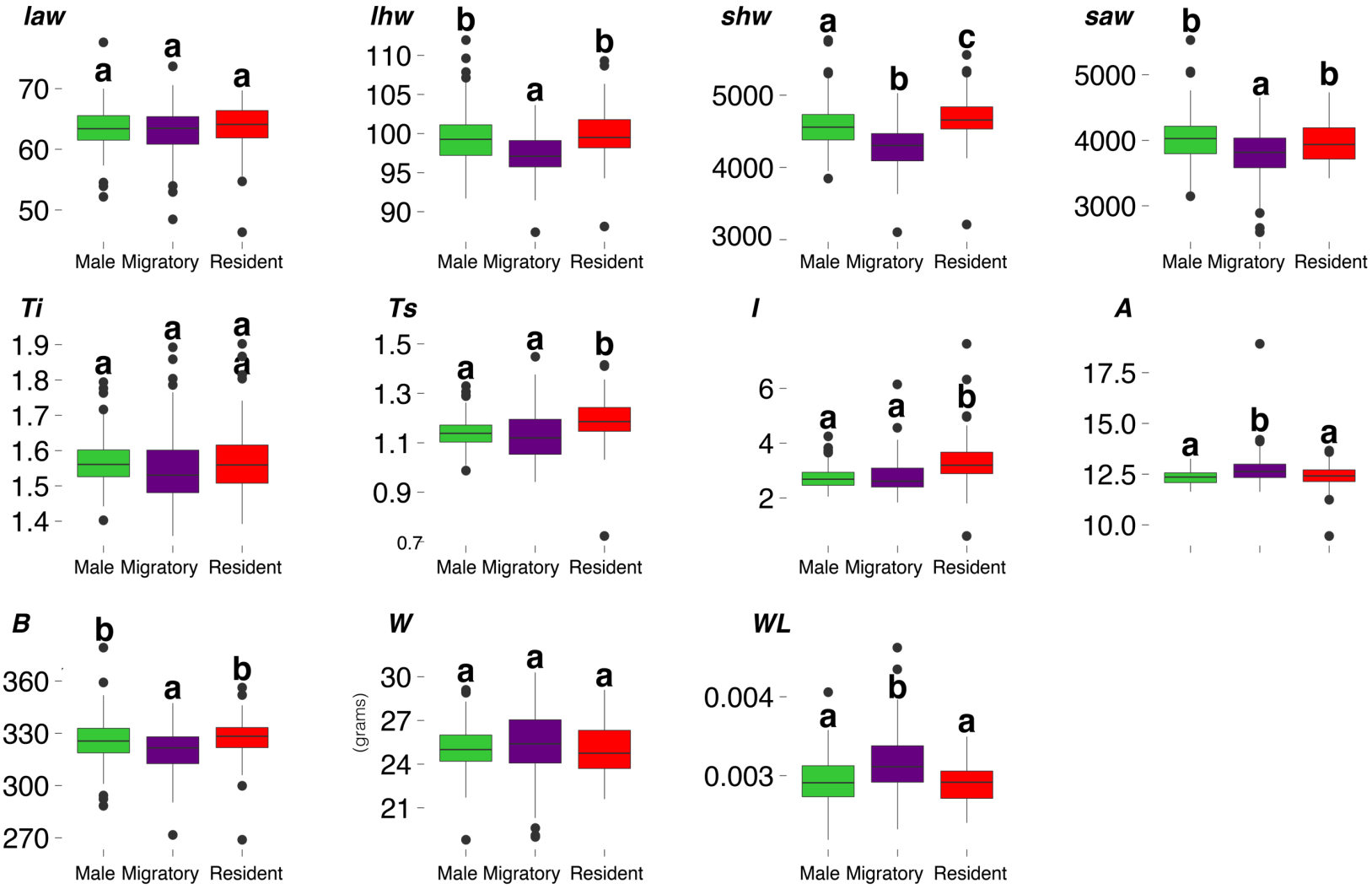


Figure 3.14 Box plots of parameters for the description of wings of *L. yerbabuenae*. Arm-wing length in mm (law), hand-wing length in mm(lhw), hand-wing area (shw), arm-wing area (saw), tip length ratio (Ti), tip area ratio (Ts), tip shape index (I), aspect ratio (A), wingspan (B), body mass in g (W), wing loading (WL). Box plots followed by a different letters are significantly different according to TukeyHSD test at a 0.01 level of significance.

3.5.7 Principal Component Analysis (PCA)

The exploratory Principal Component Analysis (PCA) revealed that the first two components described 70.32% of the variation in the data; the deformation grids with the thin-plate spline show the wing shape temporal variation (Figure 3.15). Landmarks 3 and 4 PC1 explain most of the variation in PC1 (Table 3.9), which change in the opposite direction (upwards) compared with landmarks 8 and 9 (Figure 3.15); while landmarks 1 and 2 explain most of the variation in wing shape in PC2 (Table 3.9), which move towards the centre of the wing (Figure 3.15).

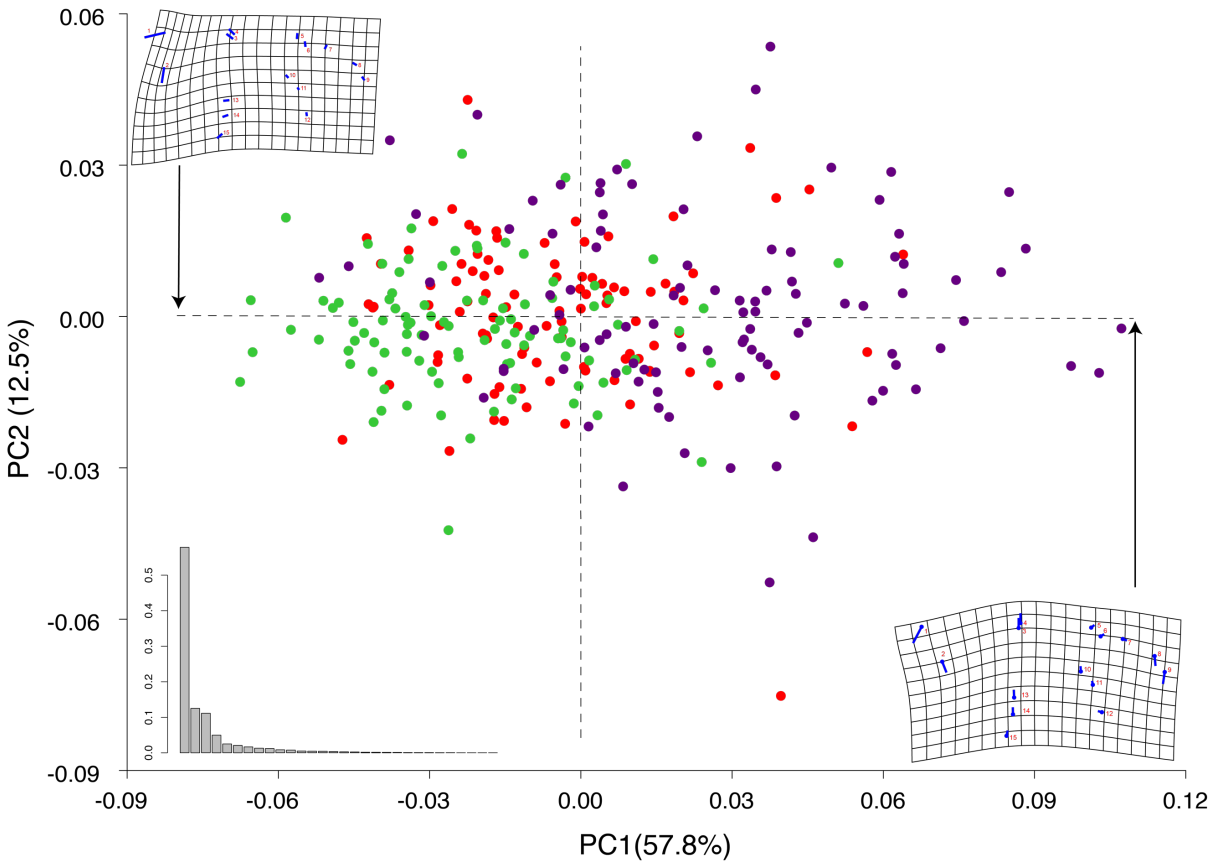


Figure 3.15 Principal Components 1 and 2 (PC) explaining 70.32% (eigenvalues) of the observed variation in wing shape of *L. yerbabuenae*. Thin-plate spline deformation grids depicting shape variation for PC1 and PC2, populations are colour coded: in green: males, purple: migratory, red: resident.

Table 3.9 Variable loadings and percent variance explained for the first five principal component axes of a PCA for 15 landmarks of 300 specimens of *L. yerbabuenae* from three populations (Male, Migratory, Resident).

Landmark coord.	*PC1*	*PC2*	*PC3*	*PC4*	*PC5*
x1	-0.263861	0.695027	0.045215	-0.074083	-0.183291
y1	-0.505032	0.162948	-0.307116	0.165642	0.255237
x2	0.127379	-0.076611	0.238673	0.047003	0.044776
y2	-0.322316	-0.500221	-0.086042	-0.183817	-0.259
x3	0.001151	-0.17917	0.164271	-0.11481	0.140707
y3	0.292696	0.137063	-0.066424	0.140597	0.052243
x4	0.000239	-0.158745	0.144146	-0.042465	0.095802
y4	0.289033	0.148616	-0.089589	0.180331	0.063453
x5	0.075354	0.010761	0.155753	-0.072515	0.245018
y5	0.062095	0.135021	-0.028375	-0.157813	0.205863
x6	0.085148	-0.014661	0.088173	-0.081532	0.173507
y6	0.053496	0.120909	-0.058504	-0.278038	0.142028
x7	0.102522	0.040811	0.064238	-0.121917	0.154065
y7	-0.037266	0.081731	0.004663	-0.272958	0.180937
x8	0.026624	0.084071	0.09169	0.171393	-0.242158
y8	-0.287238	-0.050232	0.245967	0.10638	0.098521
x9	-0.05231	0.050671	0.174719	0.411114	-0.377899
y9	-0.353642	-0.071095	0.371108	0.24413	0.137378
x10	-0.007098	-0.043509	0.008915	-0.231721	0.033274
y10	0.137631	0.04808	-0.139838	-0.089642	-0.184301
x11	-0.017009	-0.013053	-0.067702	-0.243589	-0.089039
y11	0.090894	0.020115	-0.153491	-0.094927	-0.252078
x12	-0.094338	0.007858	-0.102732	-0.162347	-0.293741
y12	0.026447	-0.059673	-0.089024	-0.111191	-0.328647
x13	-0.008832	-0.141716	-0.205684	0.009931	0.031929
y13	0.218364	-0.015575	0.063982	0.165794	-0.03587
x14	-0.004113	-0.132886	-0.334569	0.107445	0.047021
y14	0.195775	-0.047543	0.11512	0.153587	-0.020411
x15	0.029145	-0.128848	-0.465106	0.398093	0.220031
y15	0.139062	-0.110144	0.217563	0.031925	-0.055354

3.5.8 Canonical Variate Analysis (CVA)

The variance covariance matrix was used in the Canonical Variate Analysis (CVA), which maximised the separation among groups and revealed clear differences in wing shape among the three populations (Figure 3.16); with only 3.31% (p-value <0.001) of superimposition

within-groups as detected with the linear regression. The results of the Mahalanobis distances (MD) show that the wing shapes of the males and the resident females are more similar with each other than to the migratory females (Male-Resident MD= 2.697; Male-Migratory MD= 3.095; Migratory-Resident MD= 3.169; p-value<0.001). A graphical comparison of wing-shape differences is presented in (Figure 3.17), where the wings of bats belonging to the migratory group appear to be thinner when compared to the resident and male groups; and the wings of the resident group appear to be broader when compared to the migratory group and more similar to the wings of males.

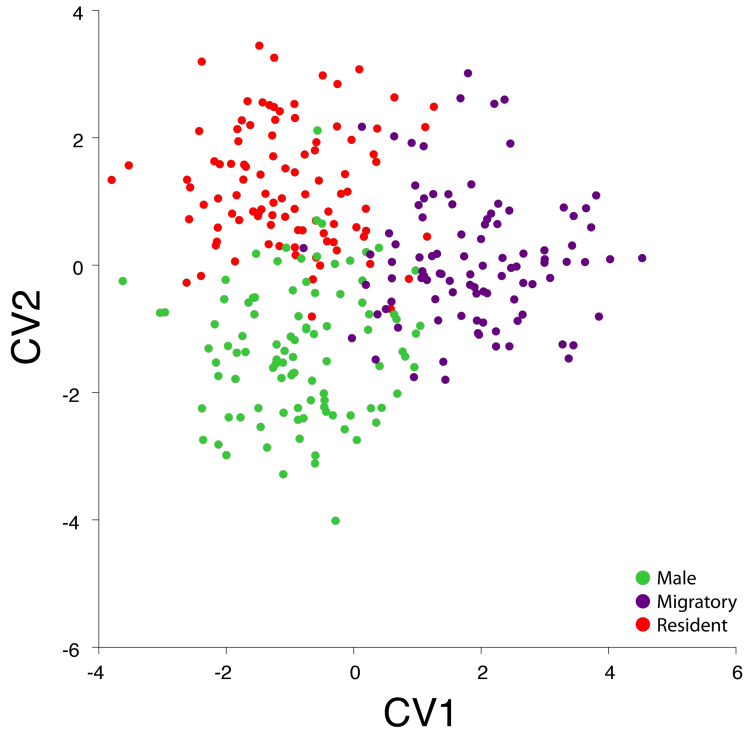
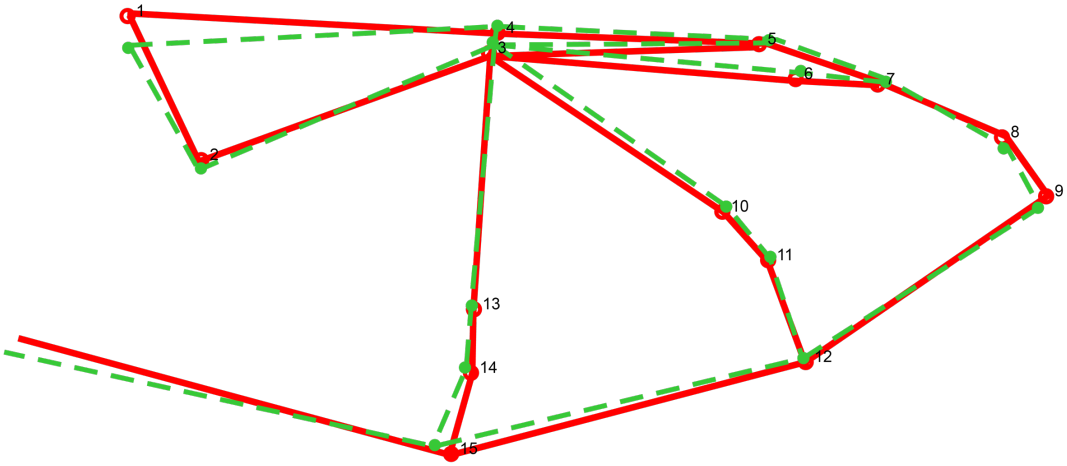
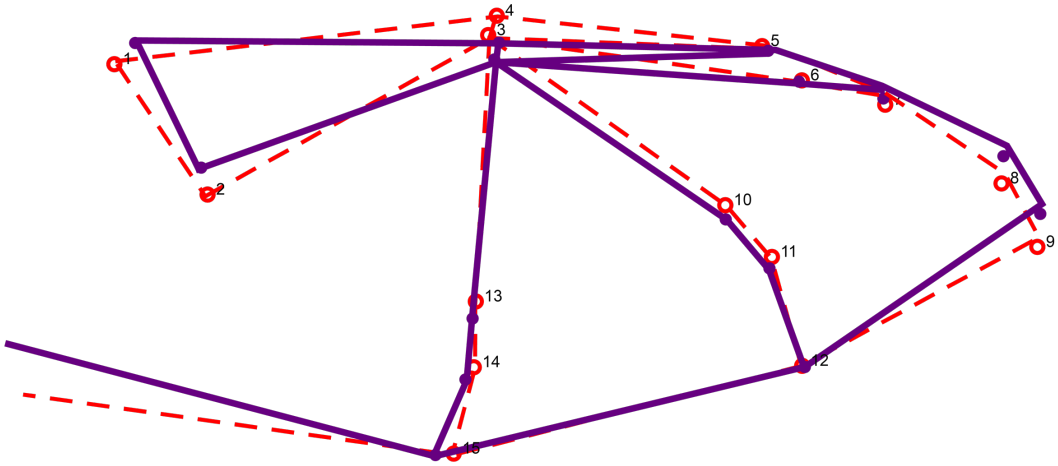


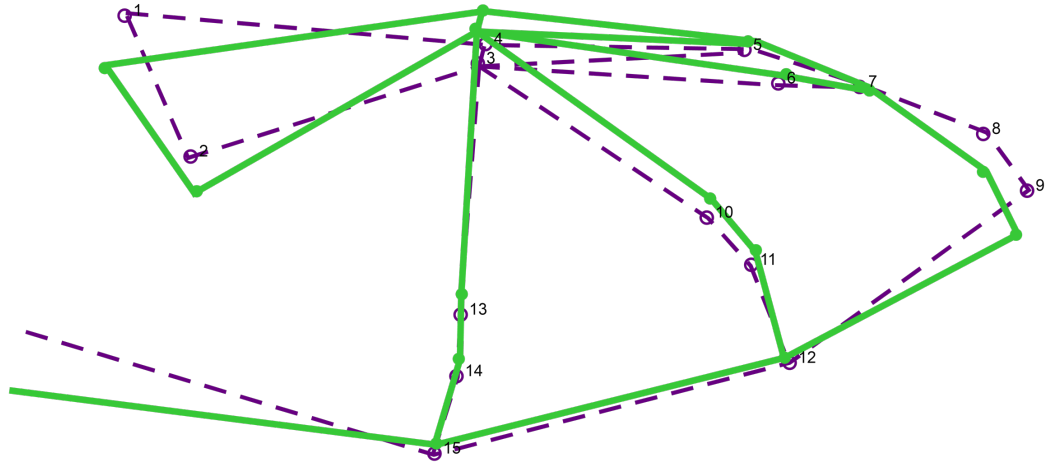
Figure 3.16 Canonical variate analysis of wing-shape changes of *L. yerbabuenae* from three populations (Male, Migratory and Resident).



Resident-Male



Migratory-Resident



Male-Migratory

Figure 3.17 Graphical comparison of wing-shape variation with a scale factor of 2x resulting from the Canonical Variate Analysis (CVA). Populations are colour coded; in green: Male, purple: Migratory, red: Resident.

3.5.9 Flight power and speed

The model predicted the most efficient flight speed to minimise energy spending per time for the migratory females at Vmp = 6.9 m/s, and that the energy expenditure would be most efficient for long distances at Vmr = 12.8 m/s. In the case of non-migratory females Vmp = 6.8 m/s and Vmr = 12.8 m/s; and for males Vmp = 6.8 and Vmr = 12.7.

3.6 Discussion

Here I present a thorough examination of the wing morphology of *L. yerbabuenae* from three populations exhibiting different migratory strategies. The comparison of 13 linear measurements and 15 landmarks from 300 bats allowed to successfully discriminate migratory from non-migratory populations based on size and shape differences. Consistent with aerodynamic theory predictions, I found that migratory females have a wing shape that is more aerodynamic and better adapted for long-distance flight with higher aspect ratio and wing loading compared to non-migratory females and males. Additionally, migratory females also have smaller hand wing area and hand-wing lengths. Interestingly, the migratory individuals had shorter wingspans, which was surprising to find as shorter wingspans are typically associated with flight in cluttered environment (Norberg and Rayner, 1987); however, shorter wingspans could be contributing to higher wing loadings especially given the higher aspect ratio observed in the migratory females. Long-distance migrants should have a high aspect ratio for flight endurance, short wings for time minimization, and a high wing loading for fast flight (Norberg, 2013).

The results of the morphological comparisons of non-migratory females and males show that both groups have wing shapes better adapted for slow speed flight and higher manoeuvrability with lower wing loading and aspect ratio than migratory females. Sexual dimorphism was only found as a difference in the length of the body which was measured with the calliper, with males being slightly larger than females by 1.8 % (Supplementary Table 3.10). Although male-biased sexual dimorphism for relative mass is widespread in Phyllostomids and has been related with large roosting aggregations and mate selection (Adams et al., 2018). In this study, I did not find evidence that males are larger than females or that all females are larger than males, more interestingly, I found many differences related to the allometry of the wing related to their migratory behaviour.

These wing shape changes are broadly explained by variation in the size of the metacarpals to the rest of the wing bones (Figure 3.7) that has an effect on the wing area. This was confirmed with the PCA of linear measurements, where the first two components explained 42 % of the variance and where the characters with the highest loadings included the metacarpals 2, 3, 4 and 5, the medial phalanx of digit 3, the forearm, and the proximal phalange of digit 4 (Figure 3.8).

A closer comparison of the variation among populations revealed that the forearm, humerus and metacarpals differ significantly among all three groups. Migratory individuals have shorter forearms and metacarpals which translates into a smaller wing area, while the non-migratory females have longer structures. Interestingly, the humerus was significantly shorter in the non-migratory populations (Figure 3.12). And overall, the wings of non-migratory females appear to be larger as observed from the size of the forearm, the metacarpals, and

the proximal phalanx of digit 3. The elongation of these hand-wing structures is what contributes to a higher hand-wing area (Figure 3.14), and which is related to slow flight speeds and where lift-generating requirements are high (Hedenström and Johansson, 2015). In contrast, the size of the humerus is significantly smaller, which could be attributed to improved hovering flight linked to movements of the humerus as observed in hummingbirds, where the position of the humerus in relation to the edge of the wing and the rotation during upstroke and downstroke allow the animal to increase the translational velocity and excursion of the wing (Warrick et al., 2012). The aerodynamic efficiency is lower for hovering than forward flight, as the body does not contribute any lift and the strong velocity of flapping does not contribute to forward speed (Håkansson et al., 2015).

I observed further population dimorphism in the wing parameters examined, with migratory females having significantly smaller arm-wing areas, hand-wing areas and hand-wing lengths than non-migratory females and males (Figure 3.14); such parameters contributed to a higher wing loading. Surprisingly the wingspan of the migratory females was significantly shorter than would be expected, but together with the rest of the wing parameters contributed to higher aspect ratios. Such features are essential for efficient steady flight as they lower the lift-induced drag linked to lift-induced vortices and produce higher power in reserve for acceleration (Norberg and Rayner, 1987). The long-distance flights that populations of *L. yerbabuenae* from northern Mexico undergo are in accord with the observed wing shape with higher aspect ratio and wing loading than the non-migratory females and males from south-central Mexico.

Furthermore, as the aspect ratio correlates with the outline of wing shape, pointier tips with higher aspect ratios and lower tip shape indices occur as the wing thins considerably towards the tip in the migratory population (Norberg and Rayner, 1987). In contrast, the non-migratory females and males show larger wing areas, lower wing loading, increased wingspan and low aspect ratio (Figure 3.14). Such parameters influence the tip shape and tip area indices, which create a more rounded wing tip, and which are critical when manoeuvring in cluttered environments (Swartz et al., 2003; Stockwell, 2001) as the lowland forests where non-migratory females inhabit in southern Mexico.

My results also show morphological variation in wing shape related to migratory strategies as observed from the geometric morphometric analysis of 15 landmarks. Similar to the results from the multivariate analysis of size changes, the components that better explained the variation of shape are the landmarks placed in the wrist bones (carpals), the humeral and forearm joints and the joint between the medial and distal phalanges of digit 3 (Table 3.9). The principal components parameters were separated by the CVA, revealing strong population dimorphism of wing shape with a higher degree of similarity between the wing shape of non-migratory males and females compared with that of migratory females (Figure 3.16). The graphical depiction of shape changes shows a broader wing for non-migratory bats and a thinner and more elongated shape for migratory bats (Figure 3.17).

Wing morphology is influenced by foraging behaviour, migratory habits and flight performance; furthermore, selection favours a more aerodynamic wing shape that is suitable for long-distance flights and favours individuals that have morphologies that increase energy efficiency and migratory success (Norberg and Rayner, 1987; Bahlman et al., 2006; Von Busse

et al., 2012), which is in accord with the observed wing morphology of *L. yerbabuenae* form northern populations; and will favour a shape suitable for hovering and manoeuvring better in dense vegetation (Stockwell, 2001; Hedenström and Johansson, 2015; Håkansson et al., 2015) as found in the non-migratory males and females of the south-central regions of Mexico.

The speed estimations of this study agree with those previously predicted for *L. yerbabuenae* (Sahley et al., 1993) and suggest that the minimum power flight speed that would keep the bats aloft for longer time is slightly higher than that of non-migratory females and males; however the maximum range speed, which is predicted to be used by migrating bats as it minimizes work done per unit distance and allow bats to fly as far as possible before exhausting fuel reserves (Holderied and Jones, 2003) was the same as non-migrating females and only slightly higher than in males. Radio tracking studies of migratory bats have estimated an average commuting speed between roosts and foraging areas at 8.9±0.9 m/s and a maximum speed of 29 m/s (Goldstein A. 2018. Pers. comm.), thus exceeding the predicted maximum speed range of *L. yerbabuenae*. Bats commuting to foraging sites from roosts are predicted to minimize commuting times in view of declining resources (Jones and Rayner, 1988). The optimal flight speed used by *L. yerbabuenae* depends on the situation that individuals are facing, my predictions are based on wing morphology and were useful to detect only very subtle variations in flight performance, however, they are more likely to reflect the speed of flight used to cover relatively short distances; future studies that follow bats along their migratory route and use high-resolution GPS tracking technology will improve our understanding of the flight behaviour in *L. yerbabuenae*. Moreover, even small distances in flight speed may result in differences of timing at stopover sites that could reflect

differences in survival probabilities or access to prime positions in nursery colonies. Natural selection is an incremental process and even the small differences predicted in flight performance could have important fitness consequences.

In the absence of gene flow, sub-populations may adapt to their local environment and exhibit genetic variance and population structuring; but if gene flow is maintained and populations are continually exchanging DNA, panmixia is likely to occur (Moussy et al., 2013). In chapter 2, I found evidence that *L. yerbabuenae* forms a single panmictic population across its range and that only very subtle genetic differentiation can be observed between the most northern and the most southern populations. Thus, we can conclude that migration has played a vital role in determining population structure and has been acting as a homogenising force in the species.

Yet, the results found in this chapter show evidence of very well-defined morphological variation in wing size and shape related to migratory strategies. If different wing shapes enable *L. yerbabuenae* to occupy different niches and to exploit different resources, how is this morphological variability possible in a population that maintains gene flow? One explanation could be that wing shape differences are due to phenotypic plasticity in response to local environmental conditions. Organisms adapting to new environments may begin with phenotypic plasticity and facilitate local adaptation that may or may not be followed by genetic changes (Ho and Zhang, 2018). Additionally, phenotypic plasticity rather than changes in a whole genotype might explain differences in gene expression among populations in different environments (Morris et al., 2014; Healy and Schulte, 2015). Alternatively, the wing shape differences may be the consequence of selection because many morphological traits

are heritable. Perhaps selection of wing shape occurs, while behavioural plasticity could be maintained by epigenetic effects that influence gene expression. In the next chapter, I continue my exploration of changes associated with the migratory strategies that *L. yerbabuenae* displays and look for variation of gene expression related to the migratory behaviour in females.

3.7 Supplementary material

Table 3.10 List of mean values with standard deviation of all traditional morphometric variables and wing parameters used for the discriminant analysis and description of the wings of *L. yerbabuenae* from three populations (Migratory, Resident and Male)

	Migratory	*Resident*	*Male*
Humerus (mm)	26.63±1.51	25.86±1.39	27.49±1.49
Forearm (mm)	53.55±1.51	55.2±1.74	53.41±1.68
Metacarpal 2 (mm)	45.98±1.58	48.01±1.68	47.26±2.28
Metacarpal 3 (mm)	52.62±1.39	54.12±1.7	53.45±2.04
Proximal phalanx digit 3 (mm)	14.22±0.6	14.86±0.67	14.42±0.66
Medial phalanx digit 3 (mm)	23.39±1.03	23.93±0.93	23.66±1.47
Distal phalanx digit 3 (mm)	12.15±1.08	12.26±0.94	12.59±0.65
Metacarpal 4 (mm)	48.5±1.39	49.9±1.56	49.15±1.78
Proximal phalanx digit 4 (mm)	11.57±0.52	11.74±0.52	11.59±0.56
Distal phalanx digit 4 (mm)	18.75±0.68	18.86±0.77	18.9±0.93
Metacarpal 5 (mm)	44.52±1.2	45.88±1.51	45.01±1.57
Proximal phalanx digit 5 (mm)	10.96±0.52	11.13±0.48	11.02±0.59
Distal phalanx digit 5 (mm)	14.62±0.65	14.65±0.63	14.62±1.07
total body length (mm)	69.96±2.6	69.6±2.46	71.24±2.56
body mass (g)	25.42±2.46	25.01±1.73	25.12±1.66
length arm wing (mm)	62.85±4.26	63.8±3.8	63.56±3.64
length hand wing (mm)	97.35±2.78	99.88±3.05	99.45±3.72
wingspan (mm)	0.32±0.01	0.33±0.01	0.33±0.01
area hand wing (mm2)	4277.6±285.61	4696.25±304.09	4586.7±338.98
area arm wing (mm2)	3797.23±386.69	3961.95±301.99	4032.16±374.52
total wing area(mm2)	16901.26±1306.35	17316.40±1023.46	17237.72±1353.36
tip length ratio	1.56±0.11	1.57±0.09	1.57±0.07
tip area ratio	1.13±0.1	1.19±0.09	1.14±0.06
tip shape index	2.77±0.6	3.31±0.85	2.72±0.4
aspect ratio	6.38±0.40	6.20±0.27	6.18±0.17
wing loading (Nm^{-2}) (Norberg & Rayner 1987)	3.17±0.42	2.9±0.25	2.94±0.33
wing loading (Norberg 1994)	10.55±1.13	9.71±0.68	9.82±0.94

Chapter 4. Comparison of blood and brain transcriptomes of the tequila bat (*Leptonycteris yerbabuenae*) in migratory and non-migratory populations

Abstract

Closely related animal species, or even populations of the same species can display surprisingly different scales and patterns of movement (Liedvogel et al., 2011). Animal migration entails morphological, physiological and behavioural adaptations that can be studied from the phenotypic to the genetic level (Lundberg et al., 2013). A substantial amount of phenotypic variation in migratory traits is heritable, but the genes shaping these phenotypes have not been identified, and differences in gene expression are also likely to be important in determining migratory predisposition (Winkler et al., 2014; Chapman et al., 2011). The tequila bat (*Leptonycteris yerbabuenae*) exhibits partial migration, with some females in southern populations migrating north to breed, while other females and males remain resident in southern and central Mexico (Wilkinson and Fleming, 1996). Here, I used next-generation sequencing to compare brain and blood transcriptomes of migratory and non-migratory bats, and identified differentially expressed genes (DEGs) that may contribute to the migratory phenotype. Enrichment analysis of brain tissue revealed relatively high expression of genes involved in well-represented pathways implicated in cognition, learning or memory, as well as locomotory exploration behaviour in migratory bats. Blood analysis revealed differences in expression of pathways associated with responses of the immune system that may confer migratory bats tolerance mechanisms for managing infection. Both tissues show high expression levels of circadian clock core genes and regulatory elements, suggesting that migration might be regulated by circadian rhythms. This is the first study to assemble and annotate blood and brain transcriptomes of *L. yerbabuenae* and to identify candidate genes that could be involved in migration.

4.1 Introduction

4.1 Migration genomics

The study of migration from a genetic viewpoint remains little researched, and has predominantly focused on variation in migratory traits in birds. For example, studies on willow warblers (*Phylloscopus trochilus*) and blackcaps (*Sylvia atricapilla*) suggest that the *Adcyap1* gene is directly associated with migratory restlessness and longer alleles have been correlated with higher migratory activity (Lundberg et al., 2013; Mueller et al., 2011). Other studies have identified circadian rhythm genes (*Clock, Npas2, Creb1, Aanat, Adcyap1, Ckiε, Period2*) as candidate genes underpinning the genetic basis of migratory behaviour in blue tits (*Cyanistes caeruleus*; Steinmeyer et al. 2009); and increased methylation at *Clock* has been proposed as a mechanism mediating phenological responses of migratory barn swallows (*Hirundo rustica*) to ongoing climate change (Saino et al. 2017).

Similarly, circadian clock genes, including a vertebrate-like cryptochrome (*Cry2*) that encodes a light sensitive protein that represses the activity of *Clock*, have been associated with sun compass orientation in monarch butterflies (*Danaus plexippus*) (Zhan et al., 2011; Reppert et al., 2010; Zhu et al., 2008). Other interesting results have been found in fish, including several species of Pacific salmonids (*Oncorhynchus spp.*), where *Clock* exhibits variation in allele frequency suggesting its involvement in mediating seasonal adaptation and influencing geographical variation in reproductive timing of highly migratory salmonids (O'Malley et al., 2010). Furthermore, gene expression analysis in the Japanese grenadier anchovy (*Coilia nasus*), revealed the involvement of olfactory pathways in the spawning migration of this species (Zhu et al., 2014).

These novel findings gave rise to the new field of "migration genomics" and have opened the door to understanding the apparent package response of migratory traits and its similarities in various taxa (Liedvogel et al., 2011). Migration in mammals however has received less attention than in birds, or in fish of economic importance; most species of mammals are terrestrial and only a small fraction of them undertake migration (Fleming and Eby, 2003).

A recent study on ungulate migration suggested that migratory behaviour may be socially transmitted. The translocation of previously migratory ungulates to new areas showed that the animals lost their migratory state and after relatively short periods (50-100 years) of learning they re-gained knowledge of habitats with more suitable forage, thus concluding that migration is a culturally transmitted behaviour that is socially learned and vertically transmitted across generations in large mammals such as the bison (*Bison bison*), the moose (*Alces alces*) and the white-tailed deer (*Odocoileus virginianus*) (Jesmer et al., 2018).

The mechanisms behind migration in bats have been proposed to be under genetic control. A study on migrating hoary bats (*Lasiurus cinereus*) tested this hypothesis by analysing the relatedness and geographical origin of migrating bats, finding no evidence of genetic relationships between sampled individuals, thus suggesting that in this species the behaviour is not socially transmitted (assuming that information transfer would occur among relatives) (Baerwald and Barclay, 2016). However, the genetic makeup behind the trait remains unknown, and information transfer is still possible among unrelated individuals.

Migratory traits may result from selection on relatively few genomic regions or loci that alter the expression levels of many genes and that can manifest in dramatic phenotypic effects (Liedvogel et al., 2011). The underlying mechanisms involving molecular, physiological or endocrinological adaptations for migration remain largely unknown and more comparative studies are necessary to broaden our understanding of the genetic architecture of migratory traits and the implications that climate change, speciation and conservation management will have on the behaviour (Mueller et al., 2011). Comparative studies of species with clear migratory divides, and particularly studies in which neutral markers have failed to explain population structure, will be useful to understand any commonalities in the migratory gene package, and will answer questions regarding which and how many genes modulate the behaviour, the role that structural protein coding genes and regulatory elements may have in controlling it, and how gene expression is influenced by the environment (Liedvogel et al., 2011).

4.1.2 Using transcriptomics to study phenotypic variation

DNA determines the identity of an organism; it is the molecule of heredity that contains the instructions for building RNA and the proteins that make up the structure of the body and carry out most of its functions (Dale 2002). While the genome contains all the genes within an organism, it is transcription of particular segments of the DNA which often determines the fate of a cell and its function (Maston et al., 2006).

The transcriptome is the set of all RNA molecules within a cell, which includes the messenger RNA (mRNA) that codes for proteins, the ribosomal RNA (rRNA) and transfer RNA (tRNA) involved in translation (Alberts et al., 2002). The RNA polymerase and other transcription

factors generate the transcriptome during transcription of the DNA; following transcription, non-coding regions (introns) are spliced to produce mature transcripts with only coding regions (exons). These transcripts then undergo translation and are the building bases of proteins (Pertea, 2012). To understand the relationship between the genome and the functioning of cells, we can study the products of the genome, specifically proteins and expressed RNAs such as tRNA and rRNA (Adams, 2008).

By measuring the intermediaries of genes and proteins (transcripts of messenger RNA) we can broaden our understanding of the genetic code, the functional mechanisms that run cells and answer questions regarding evolutionary change (Nadeau and Jiggins, 2010). Recent advances in genomic technology allows us to study patterns of variation across a range of geographical scales: contrasting neutral and adaptive variation at the genome level can be used to infer evolutionary processes driving present day population structure (Orsini et al., 2013).

By studying the transcriptome, we can gain insight of which genes are being used or turned on/off within a cell. This process starts in the early stages of development where cells differentiate to form specific tissues and continues throughout an organism's life (Alberts et al., 2002). Unlike the genome, the transcriptome changes rapidly in response to internal and external stimuli and its expression is determined by multiple factors as the stage of development of an organism, the cell type or the environmental conditions (Harrison et al., 2012). In this gene expression, a specific piece of DNA is transcribed into messenger RNA and then translated into a protein, thus expressing specific genes at any given time (Bell and Aubin-Horth, 2010).

4.1.3 RNA-seq technology to study gene expression

To study the dynamics of the transcriptome, technologies such as RNA sequencing (RNAseq) allow surveying the transcriptome in a very high-throughput and quantitative manner, and allow characterisation of the entire collection of transcripts and comparison of differential gene expression between two or more conditions (Trapnell et al., 2012).

Studying the gene expression of particular traits is also an attractive approach to study how organisms respond to the environment. For example, the study of transcriptomes with RNAseq technology has been useful to study differences in gene expression in the active and torpid states of greater horseshoe bats (*Rhinolophus ferrumequinum*) (Field et al., 2018), which suggests that neuroprotective strategies might play an important role in hibernation control mechanisms. Transcriptomics has also been used to study the responses of hibernating little brown bats (*Myotis lucifugus*) to white-nose syndrome (Field et al., 2018), and showed that torpor is a period of relative immune dormancy and arousals allow for local immune responses in infected tissues during hibernation. The use of transcriptomics has also been used to help resolve phylogenetic relationships of bats based on transcriptomic data of morphology and behaviour, supporting the classification of Yinpterochiroptera and Yangochiroptera (Lei and Dong, 2016). Transcriptomics has helped understand the genetic basis behind migration in birds, fish, and insects (Lundberg et al., 2013; Zhu et al., 2008; Cui et al., 2015; Liedvogel et al., 2011).

As seen in chapter 2, the migratory nature of one population of *L. yerbabuenae* may be responsible for the maintenance of gene flow between northern and southern populations in

Mexico, and only very subtle population structure has been observed in the species (Ramirez, 2011; Morales-Garza et al., 2007; Arteaga et al., 2018). However, this low population genetic structuring does not explain the two contrasting migratory strategies observed in the species; which as seen in chapter 3, have also influenced their wing morphology as a response to different environmental pressures. In this context, this chapter intends to answer key questions about the genetic basis of migration in *L. yerbabuenae* and will use transcriptomics to provide molecular information of the mechanisms underlying this remarkable behaviour.

4.2 Objectives

The objectives of the study are: 1) to construct a *De Novo* assembly of the transcriptome of *L. yerbabuenae;* 2) compare the gene expression of two female populations of *L. yerbabuenae* exhibiting contrasting migratory behaviours; 2) pinpoint genes associated with the migratory behaviour that might be responsive to environmental variables; and 3) explore the biological implications of the differentially expressed genes with an enrichment analysis.

4.3 Hypotheses

I hypothesise that the overall gene expression of brain and blood transcriptomes will differ substantially between migratory and non-migratory females of *L. yerbabuenae*. Such differences will be correlated to the migratory behaviour and will be useful for the identification of migratory candidate genes. As seen in other taxa, I predict that migratory bats will upregulate circadian clock genes associated with the migratory phenotype that might be responsible in triggering migratory behaviour, controlling its manifestation and/or being associated with navigation and compass orientation.

By estimating the common profile in the transcriptome and characterizing the profiles of gene expression in two different tissues, I expect to estimate the overlap of gene expression and alternative splicing in different cell profiles. Previous research has shown that some biomarkers are present in high levels in both brain and blood and that blood transcriptomes contain similar percentages of protein coding genes (Rollins et al., 2010; Worthington-Wilmer and Barratt, 1996; Schwochow et al., 2012; Huang et al., 2016). I hypothesise that the overlap between blood and brain transcriptomes will be significant, and that analysing whole blood transcriptomes will be useful for identifying migration-related genes and regulatory elements.

4.4 Methodology

4.4.1 Sample collection

Bats were captured via traditional mist netting methods (Kunz and Kurta, 1988) and samples were collected following animal welfare protocols and ethical guidelines as recommended by the guidelines of the American Society of Mammalogists (Sikes and Gannon, 2011). The necessary collection permits were obtained in accordance with The Mexican Secretariat of Environment and Natural Resources (SEMARNAT) for populations at risk or in critical habitat, permit number SGPA/DGVS/03946/15. And imported in accordance with 42 CFR Section 71.54 of the Public Health Service Foreign Quarantine Regulations issued by the Department of Health and Human Services, Centre for Disease Control and Prevention of the United States of America, permit number 2017-04-136.

The samples of migratory animals originated from the population in northern Mexico and were collected in the spring of 2016 in the Sonoran Desert when the animals arrived at their maternity roosts after migrating ~1700 km and had already given birth to their young. The non-migratory samples originated from southern Mexico and were also collected when the females were lactating, in the autumn of 2016 in a cave close to the city Tuxtla Gutierrez, Chiapas. For more details on sample localities see the methodology section of chapter 2 (Figure 2.2). Sampling in both localities was conducted after the bats returned from feeding (between 02:00-05:00 hrs); the bats were placed in bags for periods < 30min and minimal handling was attempted to prevent animals become too stressed. The animals were offered nectar and released shortly after sampling. Sample collection was done only by me, following very strict standardised protocols to avoid sample contamination.

To ensure that the RNA degraded as little as possible, the sampling took place during the last 2 days of all other sampling activities. All samples were stored in 2.0 ml criovials with 600 μl of RNA-later buffer (Thermofisher Scientific, Baltics UAB) and placed in a frozen benchtop cooler (IsoTherm-System® Eppendorf, Germany) inside an insulated picnic bag with frozen gel packs. The samples were then transported to the closest refrigerator and stored at 4°C until the end of the sampling season. When driving from one location to another, the samples were monitored with an aquarium thermometer to make sure that temperatures did not exceed 12°C. During international flights, the samples were placed inside a FRIO® insulin travel case with an ice gel pack, which could keep contents within average room temperatures (12-26°C) for over 12hrs. RNA-later buffer was preferred over flash freezing since neither liquid nitrogen nor dry ice were available near the field sites and Mexican airline guidelines forbid their use.

RNA extractions were performed within 48 hours of arriving to the NYU-Langone Medical Centre.

Blood from 80 individuals (n=40 for each site) was obtained by draining it from the antebrachial vein of each wing; the veins were first punched using sterilised insulin needles (gauge 32), and dripping blood was collected by using micropipettes with sterile tips. Each wing produced a maximum volume of ~125 ul of blood so a total of ~250 ul of blood was recovered from each specimen. Haemostatic powder was applied to the veins to prevent further bleeding. The total volume of blood recovered from each individual was <1% of its total body mass (25 g approx.), and did not exceed the recommended maximum volume that can be obtained from a small mammal (Parasuraman et al., 2010). Similar volumes of blood have been used in gene expression analysis of Myotis bats (*Myotis myotis*) and have proved to be a novel alternative to study transcriptomes in a nonlethal way (Huang et al., 2016). The brains of 4 individuals (n=2 for each site) were dissected following euthanasia by cervical dislocation; the whole organ was macerated and stored in the buffer within minutes of euthanasia. The sequences of blood and brain tissues were used to reconstruct tissue-specific transcriptomes of *L. yerbabuenae*. Other organs, including, liver, heart, eyes, tongue, spleen, lungs, kidneys, and wing tissue, were collected and stored in RNAlater buffer for potential gene expression analyses in the future.

4.4.2 RNA extraction

Total RNA was extracted from blood using the RiboPure™ RNA Purification Kit (ThermoFisher Scientific, Germany) following manufacturer's recommendations. Total RNA from brain, heart and liver was extracted using the TRIzol® LS method and treated with DNase. The overall

quality and yield of the RNA preparation was first assessed by electrophoresis on a denaturing agarose gel (Figure 4.1, A). The presence of contaminants such as proteins, salt and organic compounds were determined by measuring the absorption wavelength at A260/280 and A260/230 using a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies, Inc., Wilmington, DE, USA). The best blood samples, containing the highest yields of RNA and determined by the measurements performed with the BioAnalyzer, the agarose gel and the Nanodrop, (n=8 migratory, n=7 non-migratory) and the 4 brain samples (n=2 for each population) were further examined using an Agilent BioAnalyzer 2100 (Agilent Technologies) (Figure 4.1, B).

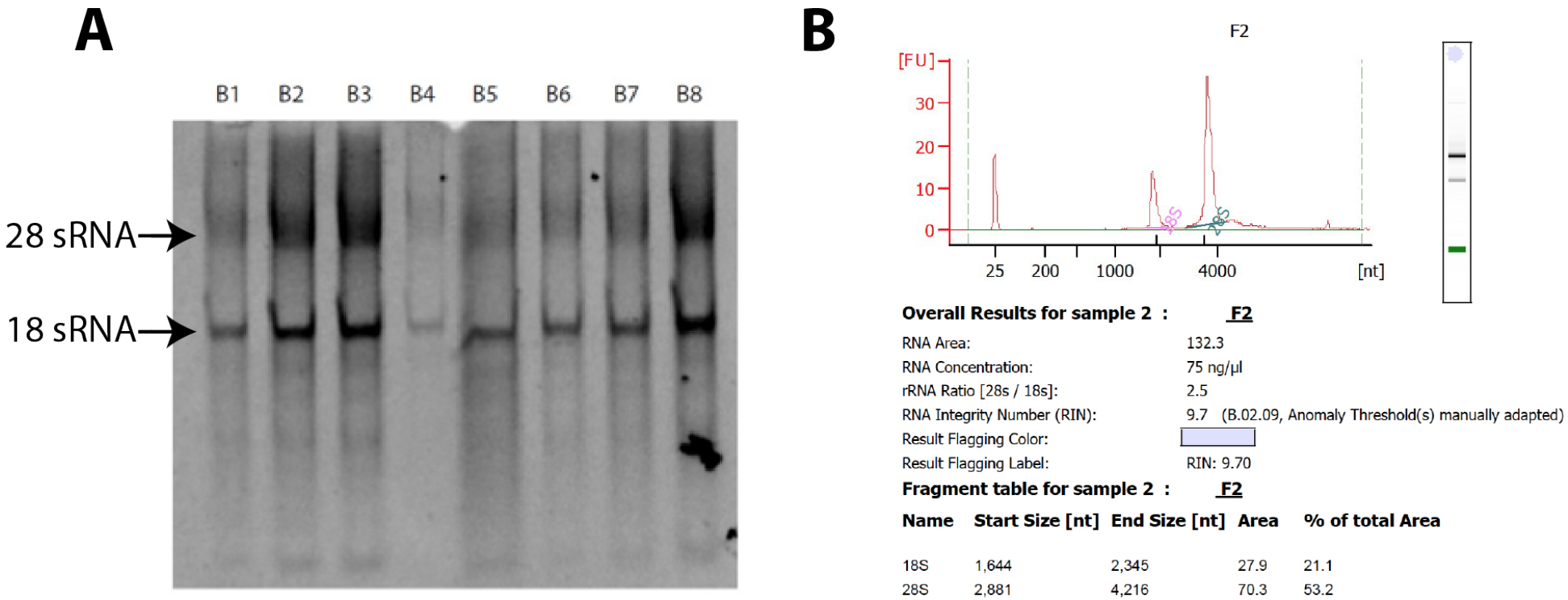


Figure 4.1 A) Visual assessment of the 28S:18S rRNA ratio on 1.5% agarose gel; the 18S and 28S ribosomal RNA bands are clearly visible in intact RNA samples; degraded RNA appears as lower molecular weight smears. Blood samples (B1-B8). B) Total RNA quality analysis depicted via Bioanalyzer; shown are representative electropherograms and gel-like images from one blood tissue preparation

4.4.3 Deep sequencing and library construction

One library was constructed for each sample; in total, 4 transcriptome-wide strand specific libraries for the brain and 15 for the blood were prepared for sequencing in four lanes in an Illumina HiSeq 2500 platform. From each sample, up to 3 strand-specific libraries were constructed: 1 for polyA+ transcripts, 1 for polyA- transcripts (both > 90 nts) and one for the small RNA fraction (between 20 and 90 nts). Strand specificity was insured by incorporating dU instead of dT in the 2nd strand and then using USER enzyme mix to remove strands before strand-specific amplification. Each library was sequenced to generate 100-bp paired-end reads on the Illumina HiSeq 2500 sequencer (v4). Sequencing yielded an average number of 203 million single-reads equivalent to 20 GB bases of single 100nt reads, and an average number of 40 million reads per sample. Library preparation and sequencing was done by the Genome Technology Centre (GTC) at the New York University Langone Medical Centre, NYU School of Medicine, USA.

4.4.4 Alignment of raw data to a known bat genome

The raw reads were first mapped to the genome of the David's myotis (*Myotis davidii*) allowing up to 3 mismatches to account for variability among species. Although more recently the vampire bat (*Desmodus rotundus)* genome has been fully sequenced and annotated (Zepeda Mendoza et al., 2018), and would have been better to use at it is also a phyllostomid, the genome of *M. davidii* was the best and more complete annotated genome at the time of the analysis. However, the vampire bat genome was used to manually annotate some of the differentially expressed genes obtained in my analysis. The similarity between the raw sequences obtained from *L. yerbabuenae* and the genome of *M. davidii* matched only by ~17 %. The low similarity in sequences was not surprising as the two species are separated at the

family level and cross-species genome mapping is highly dependent on the similarity of the genomes (Boss et al. 2016). Since the similarity between genomes was very low, I continued my analysis by doing a *De Novo* transcriptome assembly for each tissue using Trinity v. 2.4.0 (Haas et al., 2013).

4.5 *De Novo* Transcriptome assembly

This pipeline is based on the best practices for *De Novo* transcriptome assembly with Trinity proposed by Harvard University (Freedman, 2016). Bioinformatic analyses were carried out using the University of Bristol's high-performance computing (HPC) facility: Blue Crystal Phase 3. The scripts used in this analysis can be found in the supplementary material.

4.5.1 Raw data prep and clean-up

The raw RNA sequences (RNA-Seq) of each tissue were concatenated into a single file containing all left (forward) or right (reverse) reads. To assure meaningful downstream processing of the obtained raw RNA-Seq data, a quality check was performed using FastQC v 0.11.5 (Andrews, 2010) (Figure 4.2). This allowed me to check the overall quality and content of the reads, the read length, and to check for biases and sequencing artefacts, such as GC bias, read errors, primer and adapter contaminations, over represented sequences, duplicates and k-mer content. Erroneous k-mers were removed using rCorrector v 1.0.2 (Song and Florea, 2015), adapters and low-quality bases were trimmed with TrimGalore v 0.5.0 (Krueger, 2016). The sequences were mapped to the SILVA rRNA database to identify and remove small (16S/18S, SSU) and large subunit (23S/28S, LSU) ribosomal RNA (rRNA) (Quast et al., 2013; Yilmaz et al., 2014; Glöckner et al., 2017) (Figure 4.2).

5.5.2 De Novo Assembly of Transcriptome with Trinity

I ran Trinity v 2.4.0 (Haas et al., 2013) with default parameters for a stranded paired-end library with *in silico* normalization and generated N50 statistics to describe the quality of the assembly. All Trinity 'transcripts' following clustering are from here on referred as 'contigs'.

To further assess the quality of the assembly, the transcriptome was mapped using Bowtie2 v 2.2.5 (Langmead et al., 2013), and I used BUSCO v3 (Waterhouse et al., 2018) with a eukaryote dataset to evaluate the extent to which Benchmarking Universal Single-Copy Orthologs (BUSCOs) are present across higher taxonomic groupings. The redundant sequences were reduced using cd-hit v 46.8 (Li and Godzik, 2006; Niu et al., 2010) at 95% sequence identity.

4.6 Functional annotation with Trinotate

To comprehensively annotate the transcriptomes, I used Trinotate v 3.0.2 (Bryant et al., 2017) following the pipeline recommended by Harvard University (Kitzmiller, 2015) (Figure 4.2). Trinotate uses well referenced methods for functional annotations as homology search against BLAST+/SwissProt databases, protein domain identification (HMMER/PFAM) and comparison to currently curated databases as eggNOG and provides Gene Ontology terms (Kitzmiller, 2015).

Trinotate used SignalP v 4.1 (Nielsen, 2017) to predict the presence and location of signal peptide cleavage sites; TMHMM v 2.0 (Krogh et al., 2001) to predict transmembrane helices

in proteins; RNAmmer v 1.2 (Lagesen et al., 2007) to predict 5s/8s, 16s/18s and 23s/28s ribosomal RNA; HMMER v 3.1b2 (Eddy, 2018) to search databases for sequence homologs and make sequence alignments; and TransDecoder v 2.0.1 (Haas et al., 2013) to find coding regions within transcripts. I generated Venn diagrams to show the overlapping 'unigenes' annotated with Trinotate with JVenn v 1.6 (Bardou et al., 2014).

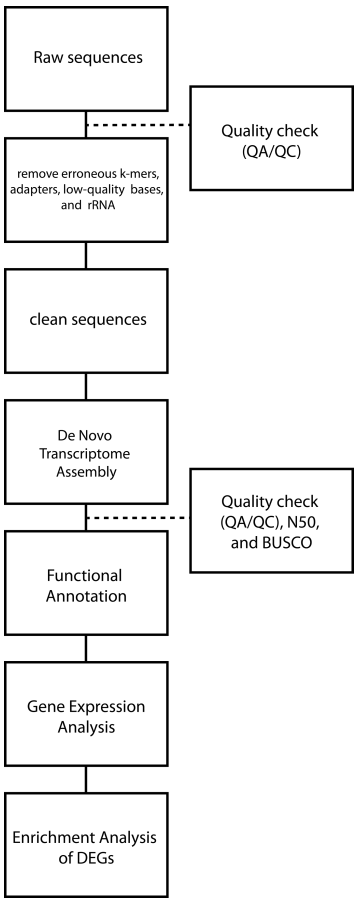


Figure 4.2 Overview of the De Novo transcriptome assembly, functional annotation, gene expression and enrichment analysis.

4.7 Analysis of gene expression

To analyse tissue-specific patterns of expression I used Partek® Flow® software v 7.0 Copyright © 2017. The pipeline includes a pre-alignment quality control (QA/QC), trimming

of low quality bases from the 3' end of reads, alignment of filtered reads using STAR v 2.4.1d, quantification to the *De Novo* assembled transcriptomes, a post-alignment QA/QC, a normalization of counts by estimating FPKM (fragments per kilobase of exon per million fragments mapped) values, an exploratory analysis using a Principal Component Analysis (PCA), and an Analysis of Variance (ANOVA) to generate a list of genes that are significantly different between migratory and non-migratory groups. The pipeline is summarized in Figure 4.3. Differentially down- and up-regulated genes were detected as those with a false discovery rate (FDR) <=0.05, p-value <= 0.05 and fold changes <-2 or >2, respectively, differentially expressed genes (DEGs) are graphically depicted in volcano plots. A list of DEGs was generated and used to explore their biological implications in a pathway enrichment analysis.

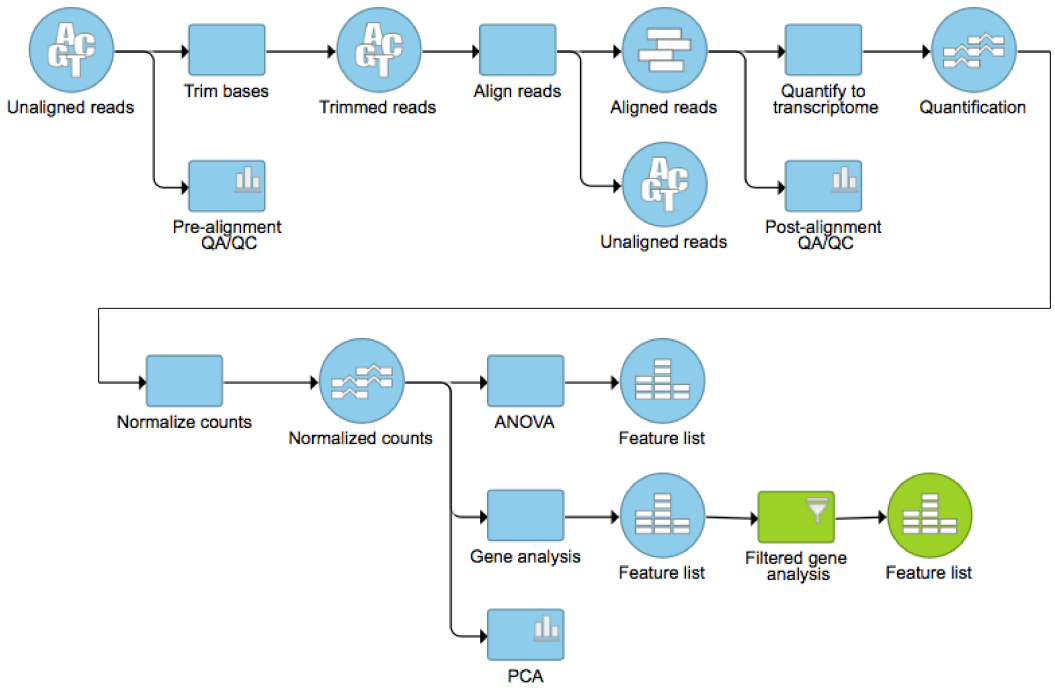


Figure 4.3 Pipeline used for the differential gene expression analysis of blood and brain transcriptomes of *L. yerbabuenae*; pipeline supported by the Partek® Flow® platform.

4.8 Enrichment analysis of DEGs

In order to explore the biological implications of the DEGs, I submitted the list of genes to Metascape (Tripathi et al., 2015) online toolkit. Metascape is a web tool that performs a workflow to analyse gene or protein lists to understand common or unique pathways and networks. The analysis was run using common mouse (*Mus musculus*) databases and categorization of pathways was done with Gene Ontology (GO) Biological Processes. Pathways with a minimum overlap of 3, a p-value cut-off of 0.01 and a minimum enrichment of 1.5 were defined as enriched.

4.9 Results

4.9.1 *De Novo* assembly

To obtain an overview of brain and blood transcriptomes of migratory and non-migratory lactating females of *L. yerbabuenae*, RNA was successfully extracted from 80 blood samples (n=40 each population) and 4 brain samples (n=2 each population). RNA integrity was assessed with a 1.5% agarose gel and with a Bioanalyzer, the best quality blood samples were chosen and 15 cDNA libraries (n=8 migratory, n=7 non-migratory) were prepared from samples of whole blood and 4 cDNA libraries from whole brain. The libraries were pair-end sequenced using an Illumina HiSeq 2500 platform and produced 110,923,465 combined raw sequences for the brain samples, and 686,474,206 sequences for the blood. After trimming adapters, low quality bases, erroneous k-mers and ribosomal RNA, the brain database contained 95,634,508 sequences and the blood database contained 570,144,922 sequences.

The high-quality reads continued in the pipeline and were used to assemble *De Novo* a brain and a blood transcriptome using Trinity. The assembly had a high coverage with an N50 median number of 1,756 bases in blood and 1,079 bases in the brain, which indicates that at least half the assembled bases are found in contigs (reads that were assembled together) whose length correspond to the N50 values (Table 4.1). The quality of the assemblies was determined by comparing them to a eukaryote dataset and evaluating the extent of presence of single copy orthologues across higher taxonomic groups; the completeness of both assemblies was deemed very high with the blood transcriptome showing 80% completeness and the brain transcriptome 94.7% (Table 4.2).

Table 4.1 Summary statistics of the De Novo transcriptome assembly of blood and brain tissues of *L. yerbabuenae*

	Blood	*Brain*
Total trinity 'genes'	111,034	417,063
Total trinity transcripts	127,369	516,584
Percent GC	54.59	47.33
Contig N10	6,831	4,954
Contig N20	4,339	3,427
Contig N30	3,162	2,453
Contig N40	2,387	1,690
Contig N50	1,756	1,079
Median contig length	365	321
Average contig	839.25	639.65
Total assembled bases	106,893,844	330,431,117

Table 4.2 Assessment of completeness of transcriptome assemblies of blood and brain tissues of *L. yerbabuenae*. Complete BUSCOs, Complete and single-copy BUSCOs, Complete and duplicated Benchmarking Universal Single-Copy Orthologs (BUSCOs), Fragmented BUSCOs, Missing BUSCOs, total BUSCO groups searched (n).

	Blood	*Brain*
Complete BUSCOs	243	287
Complete and single-copy BUSCOs	153	138
Complete and duplicated BUSCOs	90	149
Fragmented BUSCOs	50	12
Missing BUSCOs	10	4
Total BUSCO groups searched	303	303

4.9.2 Functional Annotation

Following the transcriptome assemblies, the contigs were annotated with the Trinotate pipeline. The number of contigs with 'hits' depends on which database was used, as well as on the stringency of the Basic Local Alignment Search Tool (BLAST) parameters (Altschul et al., 1997). BLAST was run against eight databases, which increased the percentage of a contig being annotated, and included SiwssProt, RNAMMER, HMMER/PFAM, SignalP, TmHMM, eggnog, KEGG, and, Gene Ontology. A summary of the annotation reports can be found in Table 4.3. The full annotation reports can be accessed through this document (https://goo.gl/LAvYLT) and include the results of the top BlastX and BlastP hit results of homology searches against the NCBI database; the RNAMMER hits show the predicted ribosomal RNA genes discovered in the assembly as predicted by hidden Markov models (HMM); the port_id, prot_coords and prot_seq provide the ID, location and translation of the longest open reading frames (ORFs); the Pfam hits represent the HMMER/PFAM protein domain search results; SignalP hits show the presence and location of predicted signal peptides; TmHMM shows the predicted transmembrane regions; eggNOG (evolutionary genealogy genes: Non-supervised Orthologous Groups) shows the results of the search of orthologous groups of genes; and finally the gene_ontology hits show the relationship of the data to Gene Ontology (GO) terms (Ghaffari et al., 2014).

The highest number of unigenes assigned with names were derived from the GO database with 46,327 brain unigenes (9%) and 10,433 blood unigenes (16%). The most represented GO categories for both tissues were cellular (GO:0005623) and intercellular components (GO:0005622). The allocated GO terms were used to classify functions based on biological processes, molecular functions, and cellular components (Figure 4.4). The Venn diagrams

shows the distribution of contigs that returned with hits against the SwissProt, eggnog, KEGG and GO databases, with a total of 2,052 overlapping unigenes in blood and 2,122 in brain (Figure 4.5).

Table 4.3 Summary of functional annotation reports generated by Trinity for the brain and blood de novo transcriptome assemblies of *L. yerbabuenae*.

	Blood	*Brain*
sprot_Top_BLASTX_hit	10,142	10,029
RNAMMER	16	22
prot_id	9,999	10,010
prot_coords	20,890	37,020
sprot_Top_BLASTP_hit	0	0
Pfam	12,062	0
SignalP	0	5
TmHMM	4,505	2
eggnog	9,050	37,511
Kegg	8,527	16,991
gene_ontology_blast	10,433	46,327
gene_ontology_pfam	12,663	17
Total contigs	95,649	516,595

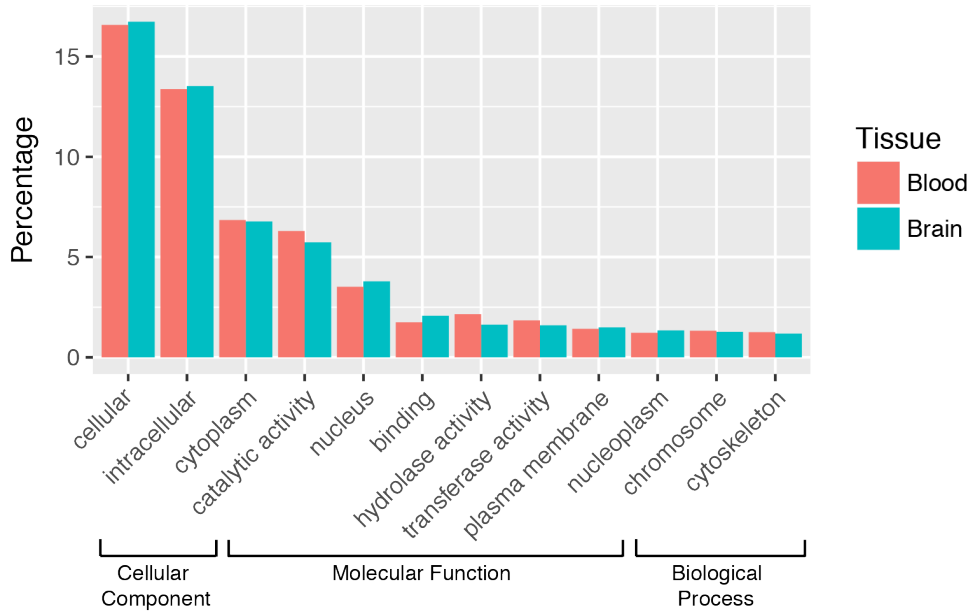


Figure 4.4 Gene Ontology (GO) classification of the twelve most represented GO categories (>1%) for blood and brain transcriptome annotations; GO terms are classified based on cellular components, molecular functions, and biological processes.

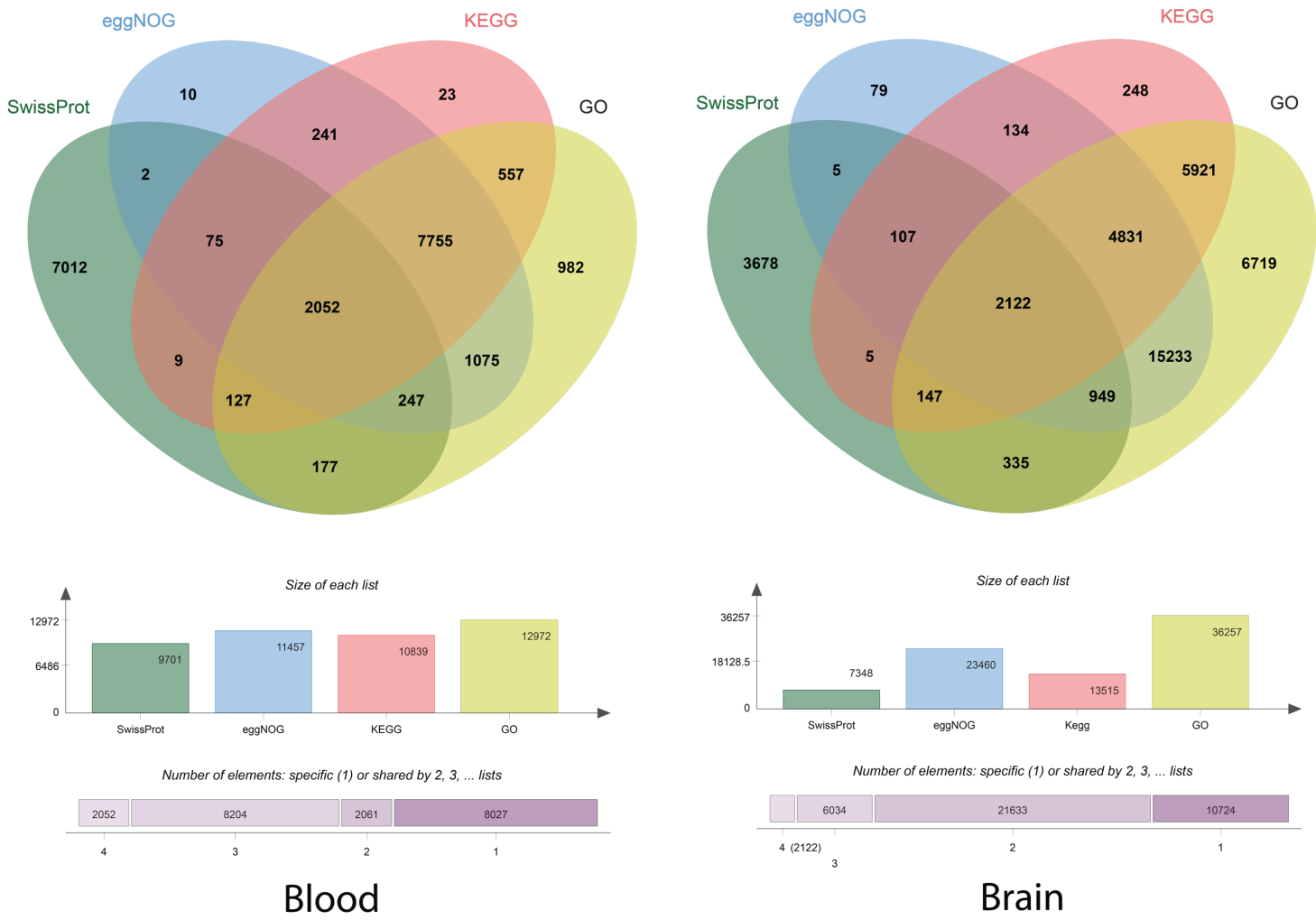


Figure 4.5 Venn diagram of shared and unique annotated contigs obtained from BLASTx against SwissProt, KEGG, eggNOG and Gene Ontology (GO) database

4.9.3 Differential expression analysis

I evaluated the number of reads assembled from each cDNA library built for each transcript and compared the patterns of gene expression patterns of migratory and non-migratory blood and brain samples. A much greater number of genes were differentially expressed in brain than in blood with the criteria of FDR <= 0.05, p-value <=0.05, and fold change <-2 or >2 (Figure 4.6). In the case of blood, all DEGs showed FDR values higher than 0.45, which reflects the high similarity of gene expression in both populations. To obtain a range of candidate genes for downstream analysis, I focused only on DEGs that showed fold changes <-1.5 and >1.5. Furthermore, the results show that there was no overlap between brain and blood DEGs suggesting tissue specificity. A list of the top ten up- and down-regulated genes in brain and blood can be found in Table 4.4.

A total of 1,955 genes were significantly differentially expressed in brain, within which 790 were up-regulated and 1,165 were down-regulated in the non-migratory state (Figure 4.6) (https://goo.gl/Yx86sU). Of these up- and down-regulated DEGs, 66.6% were annotated and among them the genes with the most significant expression difference were Dihydropyrimidinase Like 2 (*DPYSL2*) (FDR<0.02, Fold change = -858, p-value <0.0001) in the migratory state and NADH dehydrogenase subunit 3 (*NAD3*) (FDR<0.03, Fold change = -812, p-value <0.0001) in the non-migratory state. *DPYSL2* encodes a member of the collapsin mediator protein family that facilitate neuron guidance, growth and polarity (Weizmann Institute of Science, 2016), while *NAD3* codes a protein related to the mitochondrial membrane respiratory chain NADH dehydrogenase (Complex I) involved in the transfer of electrons (Weizmann Institute of Science, 2016).

In the case of blood, by looking only at differences in fold change, I obtained 397 differentially expressed genes, 85.4% of which were annotated, with 121 being up-regulated and 276 down-regulated in the non-migratory state (Figure 4.6). The genes with the highest expression differences were the Radical S-adenosyl methionine domain-containing protein 2 (*RSAD2*) (FDR=0.88, Fold change = -15.84, p-value = 0.18) in the migratory state, and the Cytidine deaminase (*CDA*) (FDR=0.73, Fold change = 4.56, p-value = 0.01) in the non-migratory state. *RSAD2* is an immune response protein that plays a major role in the cell antiviral state induced by interferons type I and II, and which can inhibit a wide range of DNA and RNA viruses (Weizmann Institute of Science, 2016). *CDA* is an enzyme involved in pyrimidine salvaging, a process where bases and nucleotides are recovered after RNA and DNA degradation (Weizmann Institute of Science, 2016).

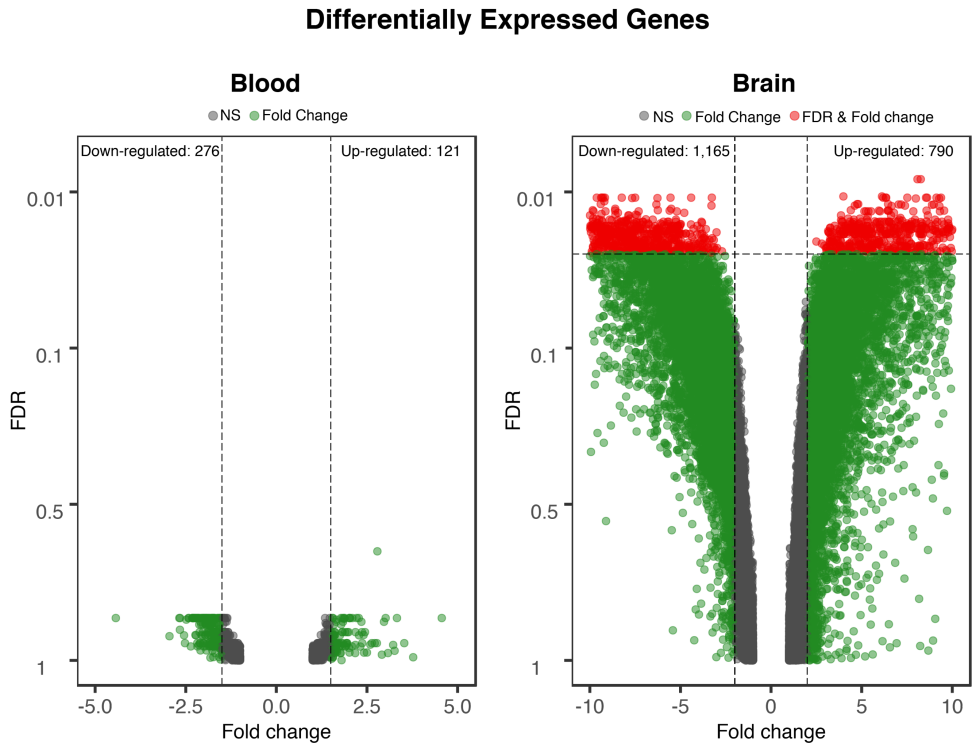


Figure 4.6 Volcano plot of differentially expressed genes in blood and brain. Dots represent single transcripts. Horizontal axis: Fold change (in log2 scale); vertical axis: FDR value. Colour coding is based on fold change (green), FDR and fold change (red), and non-significant (grey). Dotted vertical lines highlight fold changes of -2 and +2 for brain and -1.5 and 1.5 for blood. Dotted horizontal lines represent FDR value of 0.05

4.9.4 Gene Ontology Enrichment Analysis

I examined the biological process pathways that were enriched in each tissue comparison and identified a total of 464 GO categories for the migratory brain, 333 for the non-migratory brain, 424 for the migratory blood, and 169 for the non-migratory blood. Since ontology terms heavily overlap, each list was clustered by similarity and the "parent term" was explored in detail to understand the function of the genes involved. The full list of parent GO terms can be viewed in Table 4.5 and in more detail here https://goo.gl/Yx86sU).

4.9.5 Enriched pathways in the brain of migratory bats

The analysis of enriched pathways in the brain of migratory bats revealed several pathways that appear to have a function in neuronal firing and in synapse formation (Table 4.5). For example, *regulation of neuron projection development* (GO:0010975), *cellular component organization and movement* (GO:0051129, GO:0051271), or *brain development* (GO:0007420) (Figure 4.7). The importance of genes involved in the establishment of neuron networks and neuron signalling may play an important role in cognitive/behavioural differences as seen between subspecies of warblers with different migratory strategies (Boss et al., 2016); comparative studies of gene expression of warblers found an enrichment of calcium ion transport pathways in the brain during migration, which could be involved in the proliferation of neurons and in the creation of new synaptic connections necessary throughout migration (Boss et al., 2016). Furthermore, the ability to use biological compasses and maps for navigation are expected to be associated with high neuronal activity in the brain of migratory birds (Wiltschko and Wiltschko, 2014; Boss et al., 2016).

The enrichment analysis also shows well-represented GO categories that could relate to migratory behaviour (Table 4.6). These include 33 behaviour-related genes (GO:0007610) that clustered within GO groups associated with *cognition* (GO:0050890), *locomotory behaviour* (GO:0007626), *adult locomotory behaviour* (GO:0008344), *adult behaviour* (GO:0030534), and *learning or memory* (GO:0007611). Interestingly, I found enriched pathways related to *locomotory exploration behaviour* (GO:0035641) (Table 4.6, Figure 4.7).

Some of the most interesting genes in this pathway have been studied in mice and have been associated with spatial learning, anxiety, social behaviour, and reaction to novel environments. For example, the limbic system-associated membrane protein (*Lsamp*) promotes the growth of limbic neurons and guides the projections of limbic fibres; when this gene is deleted from mouse brain cells, basic neuroanatomical organization and sensory and motor development remain normal but a heightened reactivity occurs in novel environments (Catania et al., 2008).

The glutamate decarboxylase 1 (*Gad1*) encodes proteins important for the activity of GABA neurotransmitters: when mice have a deficiency in *Gad1* they show behavioural impairments in spatial learning, social behaviour, olfactory preferences and prefer familiar to novel objects (Zhang et al., 2014). The Tenascin R (*Tnr*) is a major component of the extracellular matrix of the brain that plays a role in cell differentiation, migration and communication; *Tnr*-deficient mice exhibit decreased motivation to explore and increased levels of anxiety in free choice open field tests (Freitag et al., 2003). The preproenkephalin (*Penk*) gene produces a protein involved in regulating sensory nervous system responses to external stimuli; knockout mice showed a reduction in exploratory activity in unfamiliar environments (Henry et al., 2017).

The enrichment analysis allowed me to identify genes clustered under *circadian regulation of gene expression* (GO:0032922), which included genes related to *rhythmic processes* (GO:0048511) and *circadian rhythm* (GO:0007623) (Table 4.6). The analysis also found enriched pathways related to chromatin organization (GO:0006325) (Table 4.5). Because an extensive portion of the mammalian genome is expressed in a circadian manner, chromatin remodelling has been proposed to be critical in clock function (Katada and Sassone-Corsi, 2010). Furthermore, the circadian clock has been linked to migratory timing, distance and compass orientation in birds, fish and insects (Liedvogel et al., 2011).

The classical view of the circadian clock is based on interlocked transcriptional-translational feedback loops (Dunlap, 1999). Several circadian clock genes have been identified in mammals: *Clock and Bmal1,* which act as transcriptional activators and drive the expression of *Cry1, Cry2, Per1, Per2* and *Per3* that then feedback to repress *Clock* and *Bmal1* (Katada and Sassone-Corsi, 2010). This loop drives rhythmic expression of nuclear receptors *Nr1d1* and *Nr1d2* which repress the expression of *Clock* and *Bmal1* as the second loop (Goto et al., 2017).

Two of the circadian clock genes were highly expressed in the migratory population, both are circadian repressor genes (*Per1* and *Nr1d1*). Higher levels of expression of Period 1 (*Per1*) have been observed during periods of vertical migration of zooplankton (Häfker et al., 2017), and *Per1* has also been shown play a key role in long term memory formation along with cAMP responsive element binding protein (*CREB*) in mice (Sakai et al., 2004). The nuclear receptor subfamily 1, group D, member 1 (*Nr1d1*) has been linked to circadian rhythm regulation by interacting with Period 2 (*Per2*) (Mohawk et al., 2012), and has also been shown

to modulate sociability and anxiety- related behaviour in mice (Zhao and Gammie, 2018). Experimental *Nr1d1* knockout mice display hyperactivity, impaired habituation in novel environments, deficient contextual memories and impairment in nest-building (Goto et al., 2017).

Furthermore, the enrichment analysis showed high levels of expression of seven transcription factors (*Zfhx3, CREBbp, Pdgfra, Ppargc1a, kdm5c, Ciart,* and *Gsk3b*), one neurotransmitter (*Cartpt*) and four other protein coding genes (*Arrb1, Mat2a,* and *Kmt2a*) involved in circadian clock regulation. Experimental studies have also shown that when some of these transcription factors are knocked out they alter the circadian rhythm, thus demonstrating their role as circadian oscillators. For example, the zinc finger homeobox 3 (*Zfhx3*) is a transcription factor that is highly expressed in the hypothalamus; an AT-motif functions as a clock-regulated transcriptional axis that controls the length of circadian rhythms and alters the transcriptional activity of neuropeptides in the hypothalamus. A mutation in *Zfh3* results in an acceleration of the circadian clock in mice (Balzani et al., 2016).

The peroxisome proliferative activated receptor, gamma, coactivator 1 alpha (*Ppargc1a*) is a transcriptional coactivator that regulates energy metabolism and stimulates the expression of clock genes, notably *Bmal1* and *Nr1d1.* Mice lacking *Ppargc1a* show abnormal diurnal rhythms of activity, body temperature and metabolic rate (Liu et al., 2007). CART prepropeptide (*Cartpt*) is a peptidergic neurotransmitter involved in critical biological processes such as feeding and responses to starvation (Hubert and Kuhar, 2006). An antiserum against *Cartpt* increases feeding in normal rats, indicating that *Cartpt* may be an endogenous inhibitor of food intake (Kristensen et al., 1998).

4.9.6 Enriched pathways in the blood of migratory bats

There is growing evidence that gene expression profiling of peripheral blood is a valuable tool for assessing signatures related to disease, age, response to environmental stimuli, diet, or overall health at the individual and population levels (Peters et al., 2015; Huang et al., 2016). However, the variation in gene expression patterns between migratory and non-migratory bats was very marginal in the blood samples and thus the results of the enrichment analysis should be taken lightly and we should acknowledge its limitations to draw definite conclusions regarding migration or immunity from blood samples. Still, there are interesting pathways that could be influenced by the migratory behaviour and their involvement in this mechanism cannot be ruled out.

For example, migratory bats showed evidence of highly expressed genes related to pathways of the core circadian timing machinery and genes associated with regulating the *entrainment of circadian clock by photoperiod* (GO:0043153) (Table 4.8). This GO term clustered pathways related to *photoperiodism* (GO:0009648)*, circadian regulation of gene expression* (*GO:0032922*)*, circadian rhythm* (GO:0007623), *and rhythmic process* (GO:0048511). Some of the genes found are transcriptional regulatory elements of the circadian clock that balance phosphorylation. For example, I identified the protein phosphatase 1 (*PP1*), which acts as a regulator of period and light-induced resetting of the mammalian circadian clock (Schmutz et al., 2011). The down-regulation of *PP1* by specific brain inhibitors tends to lengthen the circadian period in mice; moreover diminished *PP1* activity increases nuclear accumulation of *Per2* in neurons (Schmutz et al., 2011).

Another regulatory element, the RNA binding motif protein 4 (*Rbm4* or *Lark1*), activates the post-transcriptional expression of *Per1;* when *Lark1* is knocked down, mice have a shorter circadian period, and its overexpression results in a lengthened period (Kojima et al., 2007). O-GlcNAc transferase (*OGT*) promotes the expression of *BMAL1/Clock* target genes and also affects circadian oscillation of these core clock genes (Li et al., 2013).

Additionally, the enrichment analysis of blood shows many pathways related to regulation of the immune system (Table 4.5). For example, *T cell activation* (GO:0042110), *cytokine production* (GO:0001816), *adaptive immune response* (GO:0002250), and *T cell migration* (GO:0072678). These processes are responsible of mediating the response of the cell towards pathogens and help in the acquired immunity of the host. Interestingly, some of the genes that were highly expressed in the migratory population include interferon-induced genes (e.g. *Rsad2, Gbp1, Gbp2*); type I interferons (IFNs) are among the earliest defences against a myriad of microbes and among these *Rsad2* also known as *Viperin* (virus inhibitory protein) was involved in immunological regulatory pathways as T cell activation, lymphocyte differentiation, cytokine production, peptide secretion, adaptive immune responses, regulation of immune effector processes, symbiont processes, responses to viruses, among many others.

Enrichment of these genes associated with immune function suggests an important role in controlling viral infection and a first line of defence of the acquired immune system of migratory bats; furthermore, *Rsad2* has not only been found to restrict viral replication but is also induced by microbial products (Fitzgerald, 2011).

Additionally, natural killer cell receptors and proteins (*Nkg7* and *Cd244*) were also found to be involved in regulating several pathways of the immune system in migratory bats. Natural killer cells are only found in mammals and play a vital role in the rejection of tumorous or virally infected cells, shape the Th1 immune response, activate macrophage killing and up-regulate the expression of the major histocompatibility complex (MHC) class I (Caligiuri, 2008).

Furthermore, I found four MHC class I genes (*B*18, B*67, B*40* and *B*73*) up-regulated in the migratory population. MHC genes are closely linked to the range of pathogens that can be recognised and dealt with by the immune system and thus are important components in disease resistance. Studies on birds have proposed that a higher diversity of MHC genes may confer migratory organisms an increased tolerance to disease in face of changing environmental demands and allowing them to escape disease, staying healthy during migration (O'Connor et al., 2018); moreover, coloniality in birds has also been suggested to play a role in diversifying MHC complexes (Minias et al., 2017).

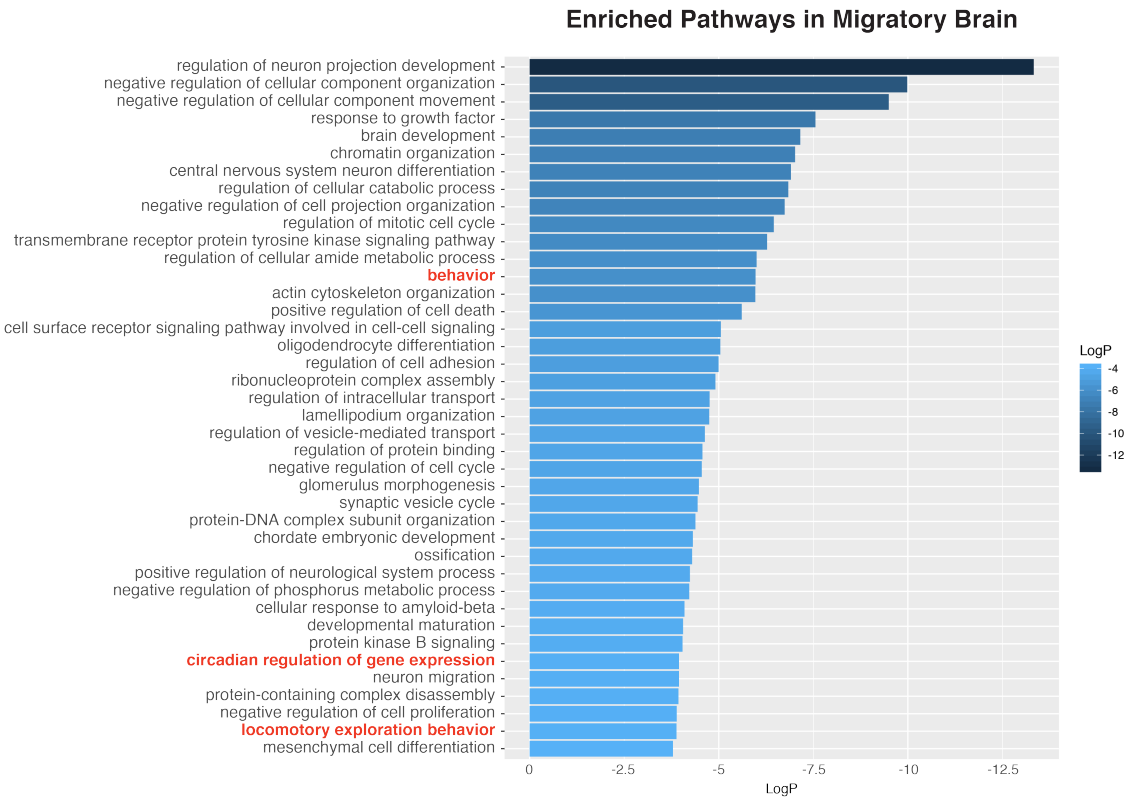


Figure 4.7 Gene Ontology (GO) of biological processes enriched across the brain of migratory bats, coloured by p-values. GO terms related to behaviour are highlighted in red.

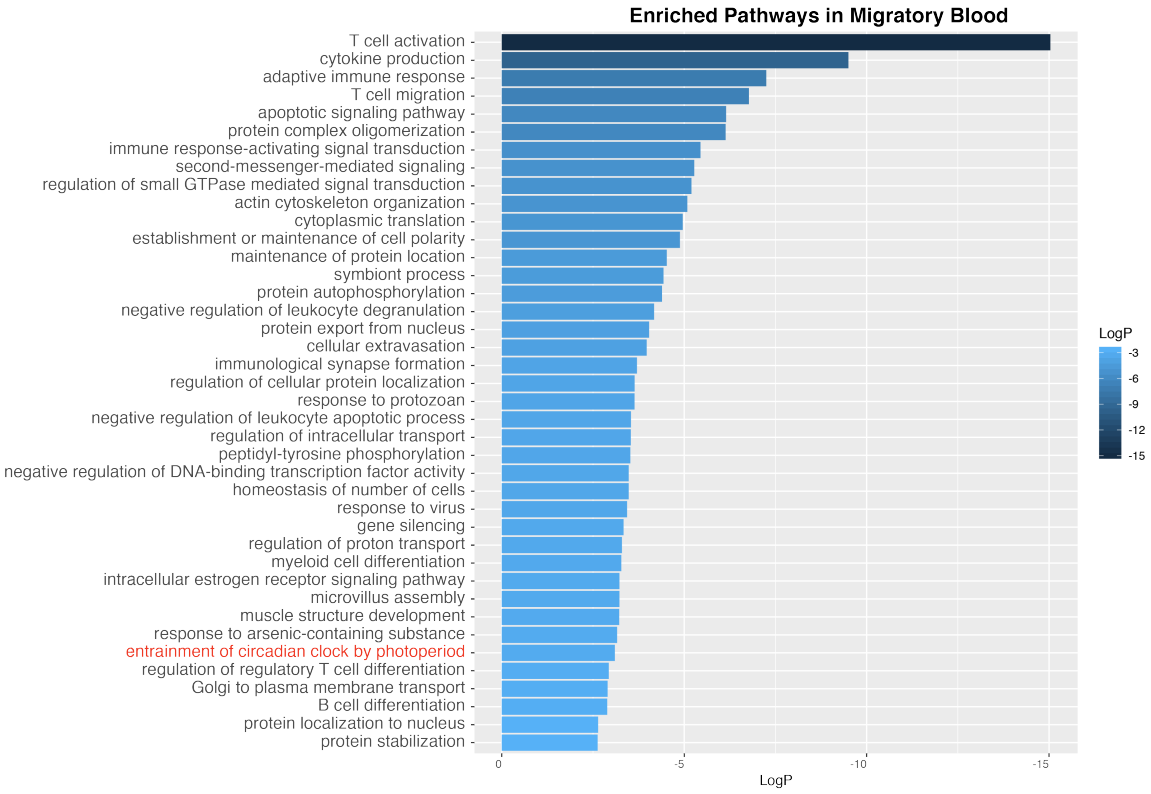


Figure 4.8 Gene Ontology (GO) of biological processes enriched across the blood of migratory bats, coloured by p-values. GO terms related to circadian rhythms are highlighted in red.

4.9.7 Enriched pathways in the brain of non-migratory bats

Some of the most interesting enriched pathways found in the brain of non-migratory bats relate to *behaviour* (GO:0007610) and *social behaviour* (GO:0035176) (Table 4.7). A total of thirty-three behaviour-related genes clustered GO groups associated with *learning or memory* (GO:0007611), and *cognition* (GO:0050890). Seven social behaviour-related genes clustered GO categories associated with *intra-species interaction between organisms* (GO:0051703), *multi-organism behaviour* (GO:0051705), *grooming behaviour* (GO:0007625), and *neuron projection arborisation* (GO:0140058). Some of the genes involved in social behaviour are related to maternal care; for example, arginine vasopressin (*Avp*) is a key neuropeptide hormone implicated in the regulation of social behaviours as maternal care and aggression; *Avp* enhances maternal behaviour and inhibits aggression in individuals exposed to chronic social stress, specially during early lactation (Coverdill et al., 2012). *Oxytocin* (*Oxt*) plays a role in maintaining social relations and allows animals which maintain cooperative relationships to show gains in longevity and offspring survival, *Oxt* has been found to increase social grooming and food sharing in the common vampire bat (Carter and Wilkinson, 2015).

Interestingly, I also found pathways related to *response to UV* (GO:0009411), which included genes related to *response to light stimulus* (GO:0009416) and *response to radiation* (GO:0009314) (Table 4.7, Figure 4.9). Some of these genes are related to responses to stress, cell protection and DNA repair. For example, *Akt1* is a serine/threonine kinase that plays a critical role in modulating cell development, growth and survival. Its expression in the nervous system has been associated with responses to cellular stress suggesting a role in cellular protection (Chog et al., 2005). *Pold1* codes a subunit of DNA polymerase delta that plays a critical role in DNA replication, it also serves to repair DNA lesions arising as result of exposure

to mutagens (Nicolas et al., 2016). This light-stimuli response in the brain of non-migratory bats may be associated with the fact that this population lives closer to an urban environment and is exposed to higher levels of artificial light and other environmental pollutants.

4.9.8 Enriched pathways in the blood of non-migratory bats

The results of the enrichment analysis of blood suggest that the immune system of this population was responding to an inflammatory process (Table 4.5). Some of the enriched pathways found in this group are related to *leukocyte migration involved in inflammatory response* (GO:0002523), some of the genes in this pathway play a role as responders of inflamed tissues (e.g. *Itgam, Gpx1*) and their expression could be related to the fact that the females were sampled shortly after parturition, which would have induced an inflammatory response and an oxidative stress response. Other pathways may also suggest that these bats were responding to external stimuli and regulating their response toward pathogens. For example, they show enrichment in pathways related to *positive regulation of immune response* (GO:0050778), *regulation of B cell mediated immunity* (GO:0002712), and *myeloid cell differentiation* (GO:0030099).

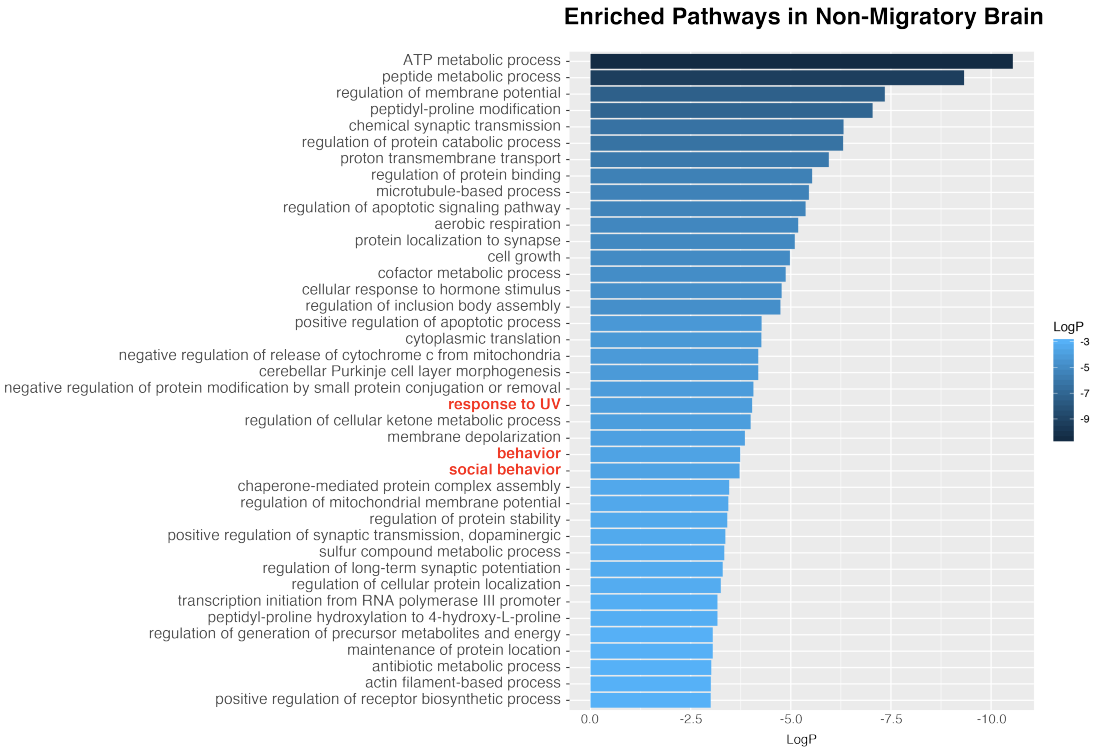


Figure 4.9 Gene Ontology (GO) of biological processes enriched across the brain of non-migratory bats, coloured by p-values. GO terms related to behaviour are highlighted in red.

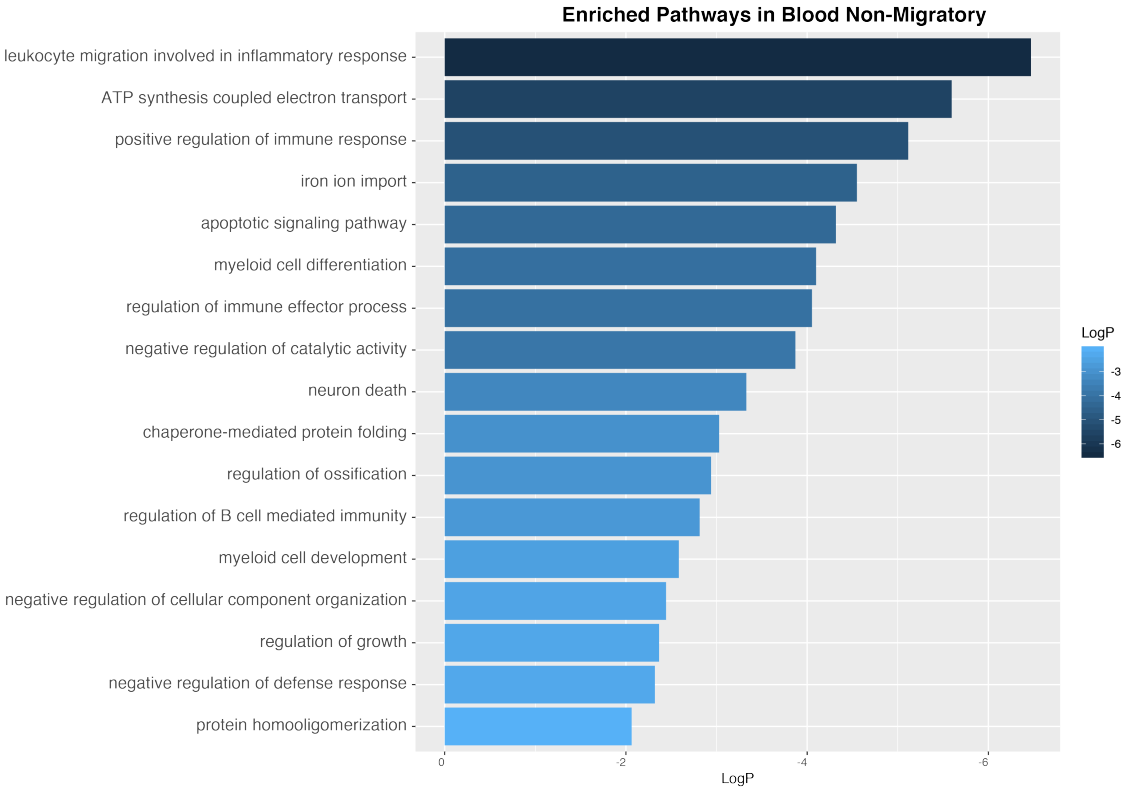


Figure 4.10 Gene Ontology (GO) of biological processes enriched across the blood of non-migratory bats, coloured by p-values.

4.10 Discussion

Here, I characterised the transcriptome of *L. yerbabuenae* for the first time and compared the gene expression of bats from two female populations of *L. yerbabuenae* exhibiting different migratory behaviours. As predicted, transcriptomics and gene expression analyses proved to be a suitable system to study genetic changes associated with migration in *L. yerbabuenae*. Although the collection permits limited the number of brain samples that I could access (n=4), and the total sample size of brain samples in this study was small compared to other gene expression studies of brain (n=6; see: Wolf et al. 2010; Lei et al. 2014; Field et al. 2018b), the overall gene expression of brain transcriptomes differed substantially between migratory and non-migratory females (Figure 4.6) and are useful to guide future studies on the genetic basis of bat migration. In the case of blood, where a bigger sample size (n=15) was studied, the differences in gene expression were marginal but show interesting aspects related to expression of genes associated with the immune system of *L. yerbabuenae*.

The results of the enrichment analysis allowed me to get a broad idea of how which genes are being up-regulated in the brain and might be directly involved in processes relevant to migration. The ones that stand out the most are related to exploration behaviour, cognition, learning and memory. Furthermore, as hypothesised, pathways related to circadian mechanisms gave me an idea of how migratory behaviour may be under the control of circadian clock genes as previously found in other migratory organisms. Although, contrary to my prediction, none of the DEGs found in brain overlapped with those found in blood, suggesting that the expression of such genes is tissue-specific, I did find regulatory elements associated with entraining circadian clock genes in blood that could indicate that different

tissues may up- or down-regulate different sets of genes which, conservatively speaking, are involved in the same pathways.

Blood was more informative on immune-related responses and may not necessarily be the best tissue to use in the study of migration from a behavioural viewpoint (Figure 4.8). However, the relatively ease of access to blood allowed me to have a greater number of samples and to prove that even with very small quantities of this tissue (250 μl or <1% of total body mass of the specimen) it is possible to obtain high yields of RNA that can be useful in immunological studies.

Explaining behaviour through genetics has been a challenging topic in biology, especially in non-model species. Compared to birds or insects, bats are phylogenetically closer to mice and humans (Lei and Dong, 2016; Tsagkogeorga et al., 2013). However, bats are still far away in the evolutionary tree of life from these organisms and the interpretation of genetic mechanisms associated with behaviour based on studies on mice should be done carefully, especially since most experimental studies are targeted towards understanding mental disorders or inherited diseases in humans and mice. For example, many of the genes that I found associated with behaviour were related to studies on autism-like disorders. Nevertheless, the vast number of behavioural studies on laboratory animals are useful to understand many of the same pathways involved in the behaviour of non-model species; in particular, experiments where specific genes are silenced, knocked out or whose function is regulated sheds light on its role and importance in a particular behaviour. The same pathways and gene functions that are very well studied in both mice and humans allows us to begin

understanding how orthologous genes and processes may be orchestrating particular behaviours in bats.

Gene expression studies provide a snapshot of what cells are responding to under particular circumstances (Ralston and Shaw, 2008). To get a better idea of which genes are more heavily related to the migratory behaviour future studies could sample individuals at different stages of their migration (before, during and after); this would allow quantification of levels of expression and would contribute to our understanding of which genes are more important for this behaviour.

The idea that a single gene governs a particular trait or that it alone determines development has long been disproven (MacKay et al., 2009). A single gene may encode proteins that play very different roles in the cell and that a single trait may be controlled by multiple genes (Paaby and Rockman, 2013). Furthermore, early life experiences can determine how genes are turned on and off and even whether some are expressed at all (Kundakovic and Champagne, 2015). We therefore need to understand organisms as self-organized systems that involve an interaction of the information they contain in their genes and information that they receive from the environment. Consequently, we cannot simply expect a single gene to explain such a complex behaviour as migration and talk about a "*migratory gene*", but we can talk about candidate genes that might have an association to migration. For the first time, this study sheds light on a set of candidate genes that may be underlying the genetic makeup behind this remarkable behaviour in a species of bat (Table 4.6, Table 4.8).

Although using whole brains can only give a gross view of what is going on in this organ, the results of the enrichment pathway analysis suggest that migratory bats build more synaptic connections and that many of these may be involved in neuron firing, which could be related to an increased ability to rapidly acquire information from the environment thus improving their cognition, memory and learning capacity (Figure 4.7). Learning and memory require neuronal activity that strengthens synaptic connections (Keverne et al., 2015). Studies on mice and rats have shown that animals living in enriched environments and exposed to novelty build more synaptic connections and are more able to acquire and use spatial information than those that are not (Speisman et al., 2013; Markham and Greenough, 2004). Learning would involve assimilating new experiences into neural networks of pre-existing knowledge and altering original networks to accommodate new information, and this neuronal firing is typically seen in laboratory animals when they learn something new (McKenzie et al., 2013). Migratory bats face daily challenges as finding stop-over sites to rest and refuel, they need to acquire new information from their environment, as well as accessing old memories, to make daily decisions to travel, find suitable roosts, food sources or to adjust in face of unexpected changes (Fleming and Eby, 2003). This degree of cognitive plasticity might explain the evolution of the migratory behaviour and would be critical to allow organisms to adapt to changing environments, especially in view of recent changes caused by climate change.

Furthermore, spatial memory is not only crucial for recognising suitable roosts and foraging locations (Carter et al., 2010); but olfactory memory also plays an important role in mother-pup recognition in free-tailed bats (*Tadarida brasiliensis*; Gustin & McCracken 1987). Olfaction has been more closely linked to memory than other senses as the olfactory bulb

and olfactory cortex output rapid neuronal connections to the hippocampus and the amygdala, which are implicated in memory processes (Mouly and Sullivan, 2010). The migratory females of *L. yerbabuenae* form large maternity colonies where young aggregate in clusters of hundreds; this behaviour is only seen in migratory *L. yerbabuenae* and may be implicated in maintaining the young at optimal developmental temperatures to ensure they will be in a suitable condition to migrate south in the autumn (Iñarritu-Castro, 2017). Memory of environmental odours and newly learned odours are crucial for survival and reproductive success in bats (see: Thies et al. 1998; Ober & Steidl 2004; Clare et al. 2011); furthermore, experimental studies have shown that mice living in odour-enriched environments exhibit better olfactory memory by maintaining an active turnover of different types of neurons and modulated by environmental signals (Rochefort et al., 2002), thus migratory bats could have improved olfactory memory associated with bulbar neurogenesis that allows them to find their pups in these large pup-aggregations.

Furthermore, the timing of migration needs to be synchronized with the flowering of plants that provide the necessary sustenance for the bats to reach their most northern roosts (Moreno-Valdez et al., 2000). In recent years, climate change has altered the phenology of plants; cold seasons, which are necessary to trigger the activity of genes related to flowering, have been altered by recent climate change causing shifts in the flowering season and also affecting the length of its duration (Chen and Penfield, 2018). Such changes in plant phenology could be detrimental to the migration of *L. yerbabuenae* if the species does not adapt to these changes fast enough (Fitzpatrick et al., 2005; Lennox et al., 2016). Epigenetic regulation of *Clock* genes has been proposed as a candidate mechanism mediating phenological responses of migratory birds to climate change (Saino et al., 2017); similar

epigenetic reprogramming would be expected in bats and may be crucial to understand how these organisms could respond to environmental variation via phenotypic plasticity.

The fact that the migratory population showed higher expression of genes related to exploration behaviour not only could explain their propensity to migrate but the potential they have to exploit new landscapes and would provide a mechanism by which migration evolved in this species and may be crucial for its survival in view of environmental change. Individuals with a higher degree of behavioural plasticity or the ability to modify their behaviour as a result of environmental inputs are more capable of adapting to novel environments than less plastic individuals (Bossdorf et al., 2008; Bjorklund, 2006). This increased adaptive potential allows organisms to enter novel environments, where they will be affected by selective pressures (Bjorklund, 2006). Furthermore, according to the Baldwin effect of adaptive learning, plastic phenotypic traits may lead to extreme outcomes where traits become genetically fixed and where environmental factors may induce the expression of pre-existing genetic variation by epigenetic processes (Sznajder et al., 2012).

The use of whole brains gave me a broad idea of the differential gene expression occurring in one of the most complex organs in the body. In this study, I macerated the entire brain and used RNAlater buffer to preserve its RNA, however flash freezing could have allowed dissecting the brain into its main components in the laboratory. Future studies should focus on looking at specific segments of the brain to have a better resolution of the gene expression of populations with different migratory strategies. For example, by looking at the pituitary gland and the hippocampus we could target genes controlling circadian rhythms (e.g. *Per1, Nr1d1*).

Another interesting approach would be looking at the gene expression of opsins both in the caudal brain region and in the retina. Opsins and cryptochromes have been associated in compass orientation and navigation using magnetic fields in birds and moths (Wiltschko and Wiltschko, 2014; Xu et al., 2017). Phyllostomid bats have S-opsin receptors that allow them to see UV light reflected from flowers (Müller et al., 2009, Simões et al., 2018); and could potentially be also involved in navigation if the reflection of UV light, which changes with the time of the day and alters the skyline, provides a visual cue for panoramic navigation as it happens in desert ants (*Melophorus bagoti*) (Schultheiss et al., 2016). Furthermore, the daily cycles in UV light levels could be responsible of entraining the circadian clock and altering the circadian rhythms as observed in the bumblebee (*Bombus terrestris*) (Chittka et al., 2013).

Further exploration of brain anatomy could focus on those regions where the greatest differences in gene expression are observed between migrants and non-migrants. The brains of migratory bats have been found to be smaller, using phylogenetically independent contrasts, than that of non-migratory ones, but the hippocampus shows no size difference (McGuire and Ratcliffe, 2011). Sagittal cuts would allow a closer inspection of the anatomy of the brain to detect subtle differences between the migratory and non-migratory brain of *L. yerbabuenae* individuals.

The pathway enrichment analyses demonstrate that both brain and blood showed relatively high expression of genes related to the regulation of the circadian clock (Table 4.6, Table 4.8). The expression of clock genes and associated regulatory elements for the coordination of internal biological processes is finely tuned with daily and seasonal light changes that

influences the expression of clock genes and regulate circadian rythms (Bell-Pedersen et al., 2005). Although my experimental design did not allow me to sample the bats at the same time of the year, given the variation of time of breeding, I did control the time of sampling to coincide with the return of the bats to their roosts after foraging and followed very strict sampling protocols. Furthermore, previous studies on candidate genes for migration have shown an association with clock genes (e.g. Häfker et al. 2017; O'Malley et al. 2010; Saino et al. 2017). Hence the results presented here support the hypothesis that migration is heavily influenced by changes in photoperiods and that core clock genes and regulatory elements play a vital role regulating this behaviour. Circadian rhythms would not only dictate the timing of migration depending on the length of the day, they need to be regulated according to the changes in day length. *L. yerbabuenae* remains in northern Mexico when the day length is ~13-14hrs between May and September, at this same time of the year the day length in central and southern Mexico is ~12.5-13hrs. Migratory bats leave northern Mexico when day length shortens to ~10-12hrs and move to south-central Mexico where the average day length is 11-12hrs. This would mean that the bats need to calibrate their circadian rhythms depending on day length. Furthermore the length of the flowering season could also be influencing the circadian clock; genetic variation in circadian genes in humans and mice is correlated with glucose homeostasis (Kalsbeek et al., 2014; Schmutz et al., 2010), and this could influence decision making on when is the best time to migrate, and would ensure that the best quality resources are available for females to feed their developing pups.

The differences found in the gene expression of blood are very marginal and cannot be used to draw big conclusions regarding the differences in the immune system of migratory and non-migratory bats, however, future studies of gene expression at finer scale may confirm

that some of the processes found in my study are relevant to immunological responses in *L. yerbabuenae* and could suggest that migratory individuals are responding to pathogens in a way that allows them to build tolerance. Furthermore, the study of immune regulatory elements may help us understand if migratory bats have developed tolerance mechanisms to manage infection as opposed to a reliance on mechanisms of host resistance to clear infections as recently discovered for the Egyptian rousette fruit bat (*Rousettus aegyptiacus*; Pavlovich et al. 2018).

It has been argued that migratory animals either lower their immunity as high-level maintenance of immune function is energetically very costly, or that they minimise the risk of disease by escaping pathogens (Buehler et al., 2010). However, migrating animals are exposed to novel environments and stressors that can cause an inflammatory response to which the immune system has to react (Costantini et al., 2018; Lennox et al., 2016). My findings support the hypothesis that the remarkable immune system of bats allows *L. yerbabuenae* to modulate interactions with pathogens and stay healthy during migration. However, to rule out alternative explanations related to variation in whole blood gene expression related to other physiological mechanisms, careful examination and analysis of positive selection of the candidate genes proposed here should take place in other migratory bats. Future studies on the diversity of MHC complexes, immunoglobulins, and natural killer cells will also expand our understanding on the nature and extent of genetic variation and immune tolerance associated with migration and would also be useful to assess the pathogen load.

The immune system of bats has attracted considerable interest recently given that bats coexist with highly pathogenic viruses without developing symptoms, and also for understanding their longevity (see: Calisher et al. 2006; Zhang et al. 2013a; Seim et al. 2013). The evolution of flight not only allowed bats to diversify and exploit new niches, but it also allowed them to deal with the by-products of an elevated metabolic rate and to develop DNA repair mechanisms that contributed to their longevity and their ability to coexist with viruses and other pathogens; this in turn must have imposed strong selective pressures on the bat genome (Munshi-South and Wilkinson, 2010; Costantini et al., 2018; Zhang et al., 2013, Kacprzyk et al., 2017). Consequently, the genes most likely to reflect the ability of bats to respond to pathogens and DNA damage caused by oxidative metabolism by-products would be those directly related to the first line of antiviral defence (Zhang et al., 2013).

Future studies that focus on specific blood cell types, for example white blood cells and the products of these cells (e.g. immunoglobulins) will allow us to better understand how the physiological stress caused by migration, the exposure to novel environments and the probability of encountering new pathogens contribute to the regulation of the immune system. The study of immunoglobulins has already proven useful to understand antibody responses of pteropodid bats to viral pathogens and their co-evolutionary relationship with viruses which may have implications in their ability to successfully cope with a diversity of viral antigens (Wynne et al., 2013; Baker et al., 2010). Furthermore, increasing attention has been paid to the importance of the microbiome and its role in health and disease (see: Phillips et al. 2012; Carrillo-Araujo et al. 2015). Weather the microbiome plays a role in pathogen inhibition or as regulator of the immune system remains unknown; studies that characterise

the microbiome during migration will shed light on how dietary changes affect microbiota communities and how this in turn could impact the immune system of migratory bats.

This is the first study to identify differential expression of candidate genes associated with migration in a species of bat, and opens the door to future research on this behaviour in other bats and mammals. Future studies on positive selection in a phylogenetic context will further confirm if the candidate genes proposed here are under selection in other migratory species and could elucidate the evolution of migration. Future studies on epigenetics (e.g. methylation, histone tail modification, noncoding RNAs) may help pinpoint the mechanisms by which early-life experiences and environmental stimuli may further affect the expression of migratory behaviour.

4.11 Supplementary tables

Table 4.4 Top 10 up- and down-regulated genes in blood and brain of non-migratory bats.

		UniprotID	*Gene Symbol*	*Protein*	*p-value*	*FDR*	*Fold change*	*LSMean non-migratory*	*LSMean migratory*
Brain	Up-regulated in non-migratory	Q38PR5	ND3	NADH dehydrogenase subunit 3	0.00	0.03	812.00	231.00	0.28
		A6QLU6	ADGRD1	Adhesion G-protein coupled receptor D1	0.00	0.03	384.00	23.60	0.06
		P48659	COX1	Cytochrome c oxidase subunit 1	0.00	0.03	375.00	1140.00	3.04
		P50679	CO2	Cytochrome c oxidase subunit 2	0.00	0.04	320.00	93.50	0.29
		P46779	RPL28	60S ribosomal protein L28	0.00	0.04	251.00	153.00	0.61
		Q9UHD8	40057	Septin-9	0.00	0.03	206.00	26.40	0.13
		Q29487	PTGDS	Prostaglandin-H2 D-isomerase	0.00	0.03	186.00	1120.00	6.03
		P04972	PDE6G	Retinal rod rhodopsin-sensitive cGMP 3',5'-cyclic phosphodiesterase subunit gamma	0.00	0.05	181.00	29.90	0.17
		Q5R844	MYL6	Myosin light polypeptide 6	0.00	0.03	181.00	52.30	0.29
		Q4R4U2	PRKCG	Protein kinase C gamma type	0.00	0.03	168.00	191.00	1.13
	Down-regulated in non-migratory	P47942	Dpysl2	Dihydropyrimidinase-related protein 2	0.00	0.02	-858.00	0.07	61.20
		P81172	HAMP	Hepcidin	0.00	0.03	-247.00	0.08	20.90
		Q8M891	ND1	NADH-ubiquinone oxidoreductase chain 1	0.00	0.02	-240.00	38.80	9310.00
		Q4R7L3	PSMC4	26S proteasome regulatory subunit 6B	0.00	0.03	-238.00	0.49	116.00
		P03920	ND5	NADH-ubiquinone oxidoreductase chain 5	0.00	0.02	-194.00	1.94	375.00
		O95197	RTN3	Reticulon-3	0.00	0.03	-164.00	0.22	36.90
		P04345	Cryga	Gamma-crystallin A	0.00	0.03	-107.00	0.17	18.00
		P00551	aphA1	Aminoglycoside 3'-phosphotransferase	0.00	0.03	-99.20	0.11	10.60

	Q37548	CO2	Cytochrome c oxidase subunit 2	0.00	0.02	-93.70	37.70	3530.00
	Q70KY4	Foxo6	Forkhead box protein O6	0.00	0.04	-72.40	0.29	21.20
Up-regulated in non-migratory	Q2HJF9	RSAD2	Radical S-adenosyl methionine domain-containing protein 2 (Viperin)	0.18	0.88	-15.84	0.79	12.46
	P02008	HBZ	Haemoglobin subunit zeta (HBAZ)	0.00	0.73	-4.43	1.13	5.03
	P30466	HLA-B	MHC class I antigen B*18	0.09	0.84	-2.95	1.61	4.75
	Q9BY19	MS4A8	Membrane-spanning 4-domains subfamily A member 8B	0.00	0.73	-2.68	2.12	5.69
	P43404	Zap70	Tyrosine-protein kinase ZAP-70	0.04	0.80	-2.66	0.93	2.47
	Q8WWW0	RASSF5	Ras association domain-containing protein 5	0.02	0.73	-2.66	1.22	3.25
	Q8NDX1	PSD4	PH and SEC7 domain-containing protein 4	0.08	0.83	-2.55	0.94	2.41
	P47967	Lgals5	Galectin-5	0.11	0.88	-2.52	6.57	16.56
	P56270	MAZ	Myc-associated zinc finger protein (MAZI)	0.02	0.74	-2.49	0.72	1.79
	Q29075	NKL	Antimicrobial peptide NK-lysine (NKL)	0.01	0.73	-2.42	1.64	3.97
Down-regulated in non-migratory	P32320	CDA	Cytidine deaminase	0.01	0.73	4.56	16.80	3.68
	Q37548	CO2	Cytochrome c oxidase subunit 2	0.89	0.98	3.78	69.75	18.47
	Q9NPB8	GPCPD1	Glycerophosphocholine phosphodiesterase GPCPD1	0.01	0.73	3.33	3.26	0.98
	Q3MI00	DNAJB1	DnaJ homolog subfamily B member 1	0.66	0.93	3.26	4.71	1.44
	Q330A8	ND2	NADH-ubiquinone oxidoreductase chain 2	0.28	0.89	3.22	77.41	24.02
	Q576B5	ND4	NADH-ubiquinone oxidoreductase chain 4	0.75	0.95	3.08	201.42	65.40
	Q7YQC6	HSPA1	Heat shock 70 kDa protein 1	0.79	0.96	2.91	6.20	2.13
	P04041	Gpx1	Glutathione peroxidase 1 (GPx-1)	0.00	0.45	2.79	3303.86	1185.50
	Q16663	CCL15	C-C motif chemokine 15	0.18	0.88	2.77	2.75	0.99
	Q9BUH6	PAXX	Protein PAXX	0.15	0.88	2.71	6.85	2.52

Table 4.5 Top 40 clusters with Gene Ontology (GO) enrichment term of Biological Process and description. “Gene count” is the number of genes found in *L. yerbabuenae* compared to all known genes in *Mus musculus* belonging to a given GO; "log10(P) is the p-value in log base 10; "Log10(q)” is the multi-test adjusted p-value in log base 10; "%" is the percentage of genes found in a given GO term.

	GO TERM	DESCRIPTION	GENE COUNT (L. YERBABUENAE / M. MUSCULUS)	LOGP	LOG(Q-VALUE)
MIGRATORY BRAIN	GO:0010975	regulation of neuron projection development	42/581	-13.33	-9.14
	GO:0051129	negative regulation of cellular component organization	41/707	-9.98	-6.83
	GO:0051271	negative regulation of cellular component movement	25/297	-9.50	-6.54
	GO:0070848	response to growth factor	33/601	-7.56	-4.91
	GO:0007420	brain development	33/625	-7.16	-4.56
	GO:0006325	chromatin organization	36/730	-7.02	-4.43
	GO:0021953	central nervous system neuron differentiation	18/217	-6.91	-4.34
	GO:0031329	regulation of cellular catabolic process	33/645	-6.84	-4.29
	GO:0031345	negative regulation of cell projection organization	17/199	-6.74	-4.21
	GO:0007346	regulation of mitotic cell cycle	29/543	-6.46	-3.96
	GO:0007169	transmembrane receptor protein tyrosine kinase signalling pathway	29/554	-6.28	-3.79
	GO:0034248	regulation of cellular amide metabolic process	22/359	-6.00	-3.57
	GO:0007610	behaviour	33/705	-5.97	-3.55
	GO:0030036	actin cytoskeleton organization	31/639	-5.97	-3.55
	GO:0010942	positive regulation of cell death	31/665	-5.61	-3.24
	GO:1905114	cell surface receptor signalling pathway involved in cell-cell signalling	25/506	-5.05	-2.77
	GO:0048709	oligodendrocyte differentiation	10/94	-5.04	-2.76
	GO:0030155	regulation of cell adhesion	29/644	-5.00	-2.72
	GO:0022618	ribonucleoprotein complex assembly	15/216	-4.91	-2.66
	GO:0032386	regulation of intracellular transport	20/367	-4.76	-2.53

	GO:0097581	lamellipodium organization	9/81	-4.75	-2.52
	GO:0060627	regulation of vesicle-mediated transport	23/470	-4.63	-2.42
	GO:0043393	regulation of protein binding	15/231	-4.57	-2.38
	GO:0045786	negative regulation of cell cycle	22/443	-4.55	-2.37
	GO:0072102	glomerulus morphogenesis	4/11	-4.47	-2.32
	GO:0099504	synaptic vesicle cycle	11/133	-4.44	-2.30
	GO:0071824	protein-DNA complex subunit organization	14/212	-4.38	-2.26
	GO:0043009	chordate embryonic development	30/738	-4.31	-2.21
	GO:0001503	ossification	19/364	-4.30	-2.20
	GO:0031646	positive regulation of neurological system process	8/73	-4.24	-2.15
	GO:0010563	negative regulation of phosphorus metabolic process	24/534	-4.22	-2.14
	GO:1904646	cellular response to amyloid-beta	5/25	-4.09	-2.05
	GO:0021700	developmental maturation	16/286	-4.05	-2.03
	GO:0043491	protein kinase B signalling	12/173	-4.04	-2.02
	GO:0032922	circadian regulation of gene expression	7/60	-3.95	-1.96
	GO:0001764	neuron migration	12/177	-3.95	-1.96
	GO:0032984	protein-containing complex disassembly	13/205	-3.93	-1.96
	GO:0008285	negative regulation of cell proliferation	27/669	-3.89	-1.93
	GO:0035641	locomotory exploration behaviour	4/15	-3.88	-1.93
	GO:0048762	mesenchymal cell differentiation	13/212	-3.79	-1.86
NON-MIGRATORY BRAIN	GO:0046034	ATP metabolic process	26/224	-10.54	-6.41
	GO:0006518	peptide metabolic process	48/746	-9.32	-6.09
	GO:0042391	regulation of membrane potential	31/428	-7.35	-4.48
	GO:0018208	peptidyl-proline modification	9/35	-7.04	-4.25
	GO:0007268	chemical synaptic transmission	42/765	-6.31	-3.59
	GO:0042176	regulation of protein catabolic process	26/357	-6.30	-3.59
	GO:1902600	proton transmembrane transport	12/88	-5.95	-3.31
	GO:0043393	regulation of protein binding	19/231	-5.53	-2.92
	GO:0007017	microtubule-based process	40/770	-5.45	-2.86
	GO:2001233	regulation of apoptotic signalling pathway	26/401	-5.37	-2.79

GO:0009060	aerobic respiration	10/71	-5.18	-2.66
GO:0035418	protein localization to synapse	8/44	-5.10	-2.59
GO:0016049	cell growth	29/500	-4.98	-2.49
GO:0051186	cofactor metabolic process	28/480	-4.87	-2.41
GO:0032870	cellular response to hormone stimulus	29/513	-4.77	-2.33
GO:0090083	regulation of inclusion body assembly	5/15	-4.74	-2.31
GO:0043065	positive regulation of apoptotic process	31/603	-4.27	-1.88
GO:0002181	cytoplasmic translation	10/90	-4.26	-1.88
GO:0090201	negative regulation of release of cytochrome c from mitochondria	5/19	-4.19	-1.83
GO:0021692	cerebellar Purkinje cell layer morphogenesis	5/19	-4.19	-1.83
GO:1903321	negative regulation of protein modification by small protein conjugation or removal	9/77	-4.07	-1.72
GO:0009411	response to UV	12/135	-4.03	-1.71
GO:0010565	regulation of cellular ketone metabolic process	11/116	-4.00	-1.69
GO:0051899	membrane depolarization	9/82	-3.85	-1.59
GO:0007610	behaviour	33/705	-3.74	-1.52
GO:0035176	social behaviour	7/51	-3.72	-1.52
GO:0051131	chaperone-mediated protein complex assembly	4/15	-3.46	-1.32
GO:0051881	regulation of mitochondrial membrane potential	8/74	-3.44	-1.31
GO:0031647	regulation of protein stability	16/253	-3.41	-1.30
GO:0032226	positive regulation of synaptic transmission, dopaminergic	3/7	-3.36	-1.27
GO:0006790	sulphur compound metabolic process	16/257	-3.34	-1.25
GO:1900271	regulation of long-term synaptic potentiation	6/43	-3.30	-1.23
GO:1903827	regulation of cellular protein localization	24/481	-3.25	-1.20
GO:0006384	transcription initiation from RNA polymerase III promoter	3/8	-3.17	-1.14
GO:0018401	peptidyl-proline hydroxylation to 4-hydroxy-L-proline	3/8	-3.17	-1.14
GO:0043467	regulation of generation of precursor metabolites and energy	9/105	-3.05	-1.04
GO:0045185	maintenance of protein location	9/105	-3.05	-1.04
GO:0016999	antibiotic metabolic process	10/128	-3.01	-1.01

	GO:0030029	actin filament-based process	31/714	-3.00	-1.01
	GO:0010870	positive regulation of receptor biosynthetic process	3/9	-3.00	-1.01
NMIGRATORY BLOOD	GO:0042110	T cell activation	28/483	-15.03	-10.84
	GO:0001816	cytokine production	26/709	-9.50	-6.67
	GO:0002250	adaptive immune response	20/557	-7.25	-4.63
	GO:0072678	T cell migration	7/49	-6.77	-4.19
	GO:0097190	apoptotic signalling pathway	19/594	-6.15	-3.62
	GO:0051259	protein complex oligomerization	19/595	-6.14	-3.62
	GO:0002757	immune response-activating signal transduction	15/427	-5.44	-3.03
	GO:0019932	second-messenger-mediated signalling	14/386	-5.27	-2.90
	GO:0051056	regulation of small GTPase mediated signal transduction	11/240	-5.20	-2.84
	GO:0030036	actin cytoskeleton organization	18/639	-5.08	-2.74
	GO:0002181	cytoplasmic translation	7/90	-4.96	-2.64
	GO:0007163	establishment or maintenance of cell polarity	10/212	-4.88	-2.59
	GO:0045185	maintenance of protein location	7/105	-4.52	-2.32
	GO:0044403	symbiont process	12/344	-4.43	-2.27
	GO:0046777	protein autophosphorylation	10/242	-4.39	-2.23
	GO:0043301	negative regulation of leukocyte degranulation	3/10	-4.17	-2.04
	GO:0006611	protein export from nucleus	7/125	-4.04	-1.93
	GO:0045123	cellular extravasation	5/56	-3.97	-1.87
	GO:0001771	immunological synapse formation	3/14	-3.70	-1.65
	GO:1903827	regulation of cellular protein localization	13/481	-3.64	-1.61
	GO:0001562	response to protozoan	4/36	-3.64	-1.61
	GO:2000107	negative regulation of leukocyte apoptotic process	5/69	-3.54	-1.54
	GO:0032386	regulation of intracellular transport	11/367	-3.53	-1.54
	GO:0018108	peptidyl-tyrosine phosphorylation	10/309	-3.52	-1.54
	GO:0043433	negative regulation of DNA-binding transcription factor activity	7/154	-3.48	-1.51
	GO:0048872	homeostasis of number of cells	10/313	-3.48	-1.51
	GO:0009615	response to virus	9/260	-3.43	-1.47
	GO:0016458	gene silencing	9/268	-3.34	-1.39

	GO:0010155	regulation of proton transport	3/19	-3.29	-1.36
	GO:0030099	myeloid cell differentiation	11/394	-3.28	-1.35
	GO:0030520	intracellular estrogen receptor signalling pathway	4/46	-3.22	-1.30
	GO:0030033	microvillus assembly	3/20	-3.22	-1.30
	GO:0061061	muscle structure development	15/674	-3.22	-1.30
	GO:0046685	response to arsenic-containing substance	3/21	-3.16	-1.26
	GO:0043153	entrainment of circadian clock by photoperiod	3/22	-3.10	-1.21
	GO:0045589	regulation of regulatory T cell differentiation	3/25	-2.93	-1.08
	GO:0006893	Golgi to plasma membrane transport	4/56	-2.90	-1.06
	GO:0030183	B cell differentiation	6/142	-2.89	-1.05
	GO:0034504	protein localization to nucleus	8/274	-2.64	-0.86
	GO:0050821	protein stabilization	6/160	-2.63	-0.85
NON-MIGRATORY BLOOD	GO:0002523	leukocyte migration involved in inflammatory response	4/17	-6.47	-2.28
	GO:0042773	ATP synthesis coupled electron transport	5/61	-5.60	-1.71
	GO:0050778	positive regulation of immune response	12/710	-5.12	-1.68
	GO:0097286	iron ion import	3/17	-4.55	-1.56
	GO:0097190	apoptotic signalling pathway	10/594	-4.32	-1.41
	GO:0030099	myeloid cell differentiation	8/394	-4.10	-1.29
	GO:0002697	regulation of immune effector process	8/400	-4.05	-1.28
	GO:0043086	negative regulation of catalytic activity	10/673	-3.87	-1.20
	GO:0070997	neuron death	7/389	-3.33	-0.90
	GO:0061077	chaperone-mediated protein folding	3/54	-3.03	-0.75
	GO:0030278	regulation of ossification	5/221	-2.94	-0.67
	GO:0002712	regulation of B cell mediated immunity	3/64	-2.81	-0.59
	GO:0061515	myeloid cell development	3/77	-2.58	-0.41
	GO:0051129	negative regulation of cellular component organization	8/707	-2.44	-0.31
	GO:0040008	regulation of growth	8/728	-2.37	-0.25
	GO:0031348	negative regulation of defence response	4/191	-2.32	-0.23
	GO:0051260	protein homooligomerization	5/355	-2.06	-0.04

Table 4.6 Most relevant Gene Ontology (GO) categories of Biological process in the brain of migratory bats. Parent GO terms are highlighted in grey.

GO	Description	Count	Gene symbols
behaviour			
GO:0007610	behaviour	33	Chrnb1,Zfhx3,Ccnd2,Clcn3,Cnp,Epha4,Epm2a,Gabrg2,Gad1,Gria1,Hipk2,Mef2c,Meis2,Mapt,Ncoa1,Penk,Ppt1,Prnp,Ptprz1,Sgk1,Thra,Tnr,Cartpt,Kmt2b,Pum1,Inpp5f,Kmt2a,Ciart,Atp1a3,Lsamp,Auts2,Foxo6,Nlgn4l
GO:0050890	cognition	19	Ccnd2,Dgcr2,Epm2a,Gnas,Gria1,Mef2c,Meis2,Mapt,Ppt1,Prnp,Ptprz1,Sgk1,Cyfip1,Thra,Tnr,Kmt2b,Kmt2a,Atp1a3,Foxo6
GO:0007626	locomotory behaviour	16	Zfhx3,Ccnd2,Clcn3,Cnp,Epha4,Gad1,Hipk2,Mapt,Penk,Ppt1,Tnr,Pum1,Inpp5f,Ciart,Atp1a3,Lsamp
GO:0008344	adult locomotory behaviour	10	Ccnd2,Clcn3,Cnp,Epha4,Hipk2,Mapt,Ppt1,Pum1,Inpp5f,Atp1a3
GO:0030534	adult behaviour	13	Chrnb1,Ccnd2,Clcn3,Cnp,Epha4,Gabrg2,Hipk2,Mapt,Ppt1,Cartpt,Pum1,Inpp5f,Atp1a3
GO:0007611	learning or memory	16	Ccnd2,Epm2a,Gria1,Mef2c,Meis2,Mapt,Ppt1,Prnp,Ptprz1,Sgk1,Thra,Tnr,Kmt2b,Kmt2a,Atp1a3,Foxo6
circadian regulation of gene expression			
GO:0032922	circadian regulation of gene expression	7	Zfhx3,Per1,Ppargc1a,Cartpt,Kmt2a,Nr1d1,Ciart
GO:0048511	rhythmic process	13	Zfhx3,Crebbp,Pdgfra,Per1,Ppargc1a,Kdm5c,Cartpt,Gsk3b,Arrb1,Kmt2a,Nr1d1,Ciart,Mat2a
GO:0007623	circadian rhythm	10	Zfhx3,Per1,Ppargc1a,Kdm5c,Cartpt,Gsk3b,Kmt2a,Nr1d1,Ciart,Mat2a
locomotory exploration behaviour			
GO:0035641	locomotory exploration behaviour	4	Gad1,Penk,Tnr,Lsamp
GO:0035640	exploration behaviour	5	Gad1,Penk,Tnr,Kmt2a,Lsamp

Table 4.7 Most relevant Gene Ontology (GO) categories of Biological process in the brain of non-migratory bats. Parent GO terms are highlighted in grey.

GO	Description	Count	Gene symbols
response to UV			
GO:0009411	response to UV	12	Akt1,Gpx1,Men1,Pold1,Rela,Sdf4,Usf1,Rev1,Cops9,Ppid,Agap3,Brsk1
GO:0009416	response to light stimulus	17	Chrnb2,Akt1,Apbb1,Nr2f6,Gpx1,Men1,Pcp2,Pold1,Rela,Sdf4,Usf1,Rev1,Rbm4b,Cops9,Ppid,Agap3,Brsk1
GO:0009314	response to radiation	20	Chrnb2,Akt1,Apbb1,Plk3,Nr2f6,Gpx1,Jun,Men1,Pcp2,Pold1,Rela,Sdf4,Usf1,Rev1,Rbm4b,Cops9,Ppid,Babam1,Agap3,Brsk1
behaviour			
GO:0007610	behaviour	33	Chrnb2,Apbb1,Aprt,Astn1,Avp,Cacna1a,Calb1,Dlg4,Dvl1,Epha4,Jun,Mef2c,Mapt,Oxt,Pak1,Penk,Prkcg,Prkcz,Prkar1b,Prnp,Ptgds,Sptbn2,Stat3,Zfp385a,Espn,Park7,Cend1,Mkks,Mapk8ip2,Myh14,Grm7,Chd7,Brsk1
GO:0007611	learning or memory	14	Chrnb2,Apbb1,Calb1,Jun,Mef2c,Mapt,Pak1,Prkcz,Prkar1b,Prnp,Zfp385a,Mapk8ip2,Grm7,Brsk1
GO:0050890	cognition	15	Chrnb2,Apbb1,Calb1,Jun,Mef2c,Mapt,Pak1,Prkcz,Prkar1b,Prnp,Zfp385a,Mapk8ip2,Grm7,Chd7,Brsk1
social behaviour			
GO:0035176	social behaviour	7	Chrnb2,Avp,Dlg4,Dvl1,Oxt,Mkks,Mapk8ip2
GO:0051703	Intra-species interaction between organisms	7	Chrnb2,Avp,Dlg4,Dvl1,Oxt,Mkks,Mapk8ip2
GO:0051705	multi-organism behaviour	8	Chrnb2,Avp,Dlg4,Dvl1,Oxt,Penk,Mkks,Mapk8ip2
GO:0007625	grooming behaviour	4	Aprt,Avp,Dlg4,Oxt
GO:0140058	neuron projection arborisation	3	Dlg4,Dvl1,Taok2

Table 4.8 Most relevant Gene Ontology (GO) categories of Biological process in the blood of migratory bats. Parent GO terms are highlighted in grey.

entrainment of circadian clock by photoperiod			
GO:0043153	entrainment of circadian clock by photoperiod	3	Ppp1cb,Ppp1cc,Rbm4
GO:0009648	photoperiodism	3	Ppp1cb,Ppp1cc,Rbm4
GO:0009649	entrainment of circadian clock	3	Ppp1cb,Ppp1cc,Rbm4
GO:0032922	circadian regulation of gene expression	4	Ppp1cb,Ppp1cc,Rbm4,Ogt
GO:0007623	circadian rhythm	6	Prf1,Ppp1cb,Ppp1cc,Ptger4,Rbm4,Ogt
GO:0048511	rhythmic process	7	Rack1,Prf1,Ppp1cb,Ppp1cc,Ptger4,Rbm4,Ogt
GO:0043255	regulation of carbohydrate biosynthetic process	4	Cd244a,Ppp1cb,Ptger4,Ogt

4.12 Bioinformatic pipelines

4.12.1 Transcriptome assembly of blood and brain from *Leptonycteris yerbabuenae*

Based on the Best Practices proposed by Harvard https://informatics.fas.harvard.edu/best-practices-for-de-novo-transcriptome-assembly-with-trinity.html (Freedman, 2016)

Data prep and clean-up:

1. All Forward and Reverse RAW sequences were merged together to have a single file containing all left or right reads.

```
cat *_R1_001.fastq.gz > ALL_R1_2017.fastq.gz
cat *_R2_001.fastq.gz > ALL_R2_2017.fastq.gz
```

2. A Quality Control was performed using ***FASTQC*** to check the overall quality and content of the reads, read length, k-mer content, the presence of ambiguous bases, over-represented sequences, and duplicates. (30 min)

```
module add apps/fastqc-0.11.5
fastqc ALL_R*_Blood.fastq.gz
```

3. The generated HTML files were transferred into the desktop to review the results.

```
scp am14886@137.222.168.10:/home/am14886/blood/ALL_R*_Blood_fastqc.html .
```

4. Erroneous k-mers were removed from the pair-reads using **rCorrector** (10 hrs)

```
git clone git@github.com:mourisl/Rcorrector.git
cd Rcorrector-master/
mkdir

#!/bin/bash
# request sources:
#PBS -l nodes=1:ppn=10,walltime=100:00:00

CD $PBS_O_WORKDIR
perl run_rcorrector.pl -1 /panfs/panasas01/bisc/am14886/Blood/all_blood_R1.fastq.gz
-2 /panfs/panasas01/bisc/am14886/Blood/all_blood_R2.fastq.gz
```

5. Discard read pairs for which one of the reads is deemed unfixable using **FilterUncorrectablePEfastq.py** (< 8hrs)

```
https://github.com/harvardinformatics/TranscriptomeAssemblyTools.git

#!/bin/bash
# request sources:
#PBS -l nodes=1:ppn=10,walltime=100:00:00

CD $PBS_O_WORKDIR
module load  languages/python-2.7.10
python FilterUncorrectabledPEfastq.py -1
/panfs/panasas01/bisc/am14886/all_blood_R1.cor.fq.gz  -2
/panfs/panasas01/bisc/am14886/all_blood_R2.cor.fq.gz -o fixed 2>&1 >
rmunfixable_blood.out
```

6. Trim adapter and low quality bases from fastq files with **Trim Galore** (1min)

```
wget
http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/trim_galore_v0.4.1.zi
p
unzip trim_galore_v0.4.1.zip
```

install cut adapt:

```
pip install --user --upgrade cutadapt
```

get path to cut adapt with:

```
which cutadapt
```

```
#!/bin/bash
# request sources:
#PBS -l nodes=1:ppn=10,walltime=100:00:00


CD $PBS_O_WORKDIR
/panfs/panasas01/bisc/am14886/trim_galore_zip/trim_galore --path_to_cutadapt
/cm/shared/languages/anaconda2-5.0.1/bin/cutadapt --paired --retain_unpaired --
phred33 --output_dir trimmed_reads --length 36 -q 5 --stringency 1 -e 0.1
/panfs/panasas01/bisc/am14886/fixed_all_blood_R1.cor.fq
/panfs/panasas01/bisc/am14886/fixed_all_blood_R2.cor.fq
```

7. Map reads to the SILVA rRNA database

7.1 download the SILVA database

```
https://www.arb-silva.de/no_cache/download/archive/release_132/Exports/
```

7.2 Build index with Bowtie2

```
bowtie2-build -f SILVA_132_SSURef_Nr99_tax_silva.fasta SILVA_index
```

7.3 Align sequences to index (7 hrs)

```
#!/bin/bash
# request sources:
#PBS -l nodes=1:ppn=10,walltime=100:00:00

bowtie2 --very-sensitive-local --phred33 --nofw  -x
/panfs/panasas01/bisc/am14886/SILVA_index -1
/panfs/panasas01/bisc/am14886/fixed_all_blood_R1.cor.fq  -2
/panfs/panasas01/bisc/am14886/fixed_all_blood_R2.cor.fq --threads 12 --met-file
summary_SILVA --al-conc-gz mapped_reads.fq.gz --un-conc-gz
mapped_reads_unmatched.fq.gz --al-gz mapped_reads_mapped_single_end.fq.gz --un-gz
mapped_reads_unmapped_single_end.fq.gz
```

Reads that will continue in the pipeline: `mapped_reads_unmatched.*.fq.gz`
To uncompress the file: `zcat mapped_reads_unmatched.2.fq > mapped_reads_unmatched_unzip.2.fq`

8. Run FastQC on processed reads

```
module add apps/fastqc-0.11.5
fastqc mapped_reads_unmatched.fq.*.gz
```

9. BLAST the over-represented sequences to see what they are

10. OPTIONAL remove remaining over-represented sequences.

```
use RemoveFastqcOverrepSequenceReads.py
```

10. Count the number of sequences in your filtered files for a quick comparison.

```
for .fa files use:
grep -c "^>" reads.fa

for .fq or fq.gz use:
zcat reads.fq.gz | echo $((`wc -l`/4))
```

4.12.2 De Novo Assembly of Transcriptome with Trinity

1. Assemble the transcriptome using trinity with normalisation (24-48hrs).

```
#!/bin/bash
#PBS -q himem
#PBS -l nodes=1:ppn=16
#PBS -l walltime=200:00:00
```

```
cd /panfs/panasas01/bisc/am14886/
# run program

Trinity --seqType fq --SS_lib_type RF --trimmomatic --normalize_reads --left
mapped_reads_unmatched.1.fq --right mapped_reads_unmatched.2.fq --output
trinity_transcriptome_blood_NorRNA --CPU 16 --max_memory 240G
```

2. Generate N50 statistics and counts of the total contigs generated during the assembly (5min)

```
~/Trinity-v2.6.5/util/TrinityStats.pl trinity_assembly_NoAdapters.fasta >
trinity_assembly2.metrics
```

3. Quantify read support of the assembly by mapping them with Bowtie2

3.1 Build a bowtie2 index for the assembly

```
module add apps/bowtie-2.2.9
bowtie2-build -f trinity_assembly_NoAdapters.fasta NoAdapters_index
```

3.2 Map reads to the index

```
#!/bin/bash
# request sources:
#PBS -l nodes=1:ppn=10,walltime=100:00:00

bowtie2 -p 16 --local --no-unal -x /panfs/panasas01/bisc/am14886/NoAdapters_index -
q -1 mapped_reads_unmatched.1.fq.gz -2 mapped_reads_unmatched.2.fq.gz | samtools
view -Sb - | samtools sort -no - - > bowtie2.nameSorted.bam
```

3.3 Generate alignment statistics

```
~/Trinity-v2.6.5/util/misc/SAM_nameSorted_to_uniq_count_stats.pl
bowtie2.nameSorted.bam
```

4. Assess the completeness of the assembly using BUSCO (5min)

```
BUSCO was added to my environment in:  languages/python-anaconda-5.0.1-2.7

BUSCO.py -c 16 --in trinity_assembly_NorRNA.fasta --out NorRNA_busco -l
eukaryota_odb9 --mode tran
```

5. Filter sequences by a 95% similarity threshold. (hrs)

```
#!/bin/bash
# request sources:
#PBS -l nodes=1:ppn=10,walltime=020:00:00

cd-hit -i brain_transcriptome.fasta -c 0.95 -o brain_transcriptome_cdhit.fasta
```

4.12.3 Functional Annotation and Analysis with Trinotate

Pipeline recommended by Harvard University (Kitzmiller, 2015).

1. Generate protein profiles to be used by Trinotate

```
~/Trinotate-3.0.2/admin/Build_Trinotate_Boilerplate_SQLite_db.pl Trinotate
```

2. Prepare protein database for Blast search:

```
makeblastdb -in uniprot_sprot.pep -dbtype prot
```

3. Uncompress and prepare the Pfam database for use with **hmmscan**

```
gunzip Pfam-A.hmm.gz
hmmpress Pfam-A.hmm
```

4. Create a file of the most likely Longest-ORF peptide candidates generated from the Trinity Assembly with **transdecoder**. (45 min)

```
TransDecoder.LongOrfs -t trinity_assembly_NorRNA.fasta
TransDecoder.Predict -t trinity_assembly_NorRNA.fasta
```

5. Capture BLAST homologies of Trinity transcripts (**BLASTX)** (50-75 hrs)

```
#!/bin/bashqs
# request resources:
#PBS -l nodes=1:ppn=10,walltime=100:00:00

cd $PBS_O_WORKDIR

# search transcript nucleotides

blastx -query /panfs/panasas01/bisc/am14886/trinity_assembly_NorRNA.fasta -db
/panfs/panasas01/bisc/am14886/uniprot_sprot.pep -num_threads 8 -max_target_seqs 1 -
outfmt 6 > blastx.blood.outfmt6
```

6. Capture BLAST homologies of Transdecoder predicted proteins **(BLASTP)** (50-100 hrs)

```
#!/bin/bash
# request resources:
#PBS -l nodes=1:ppn=10,walltime=100:00:00

cd $PBS_O_WORKDIR

# search transcript peptides

blastp -query /panfs/panasas01/bisc/am14886/trinity_assembly_NorRNA.fasta  -db
/panfs/panasas01/bisc/am14886/uniprot_sprot.pep -num_threads 8 -max_target_seqs 1 -
outfmt 6 > blastp.blood.outfmt6
```

7. Identify protein domains with **HMMER** (9 hrs)

```
#!/bin/bash
# request resources:
#PBS -l nodes=1:ppn=8,walltime=100:00:00

cd $PBS_O_WORKDIR

#hmmscan (HMMER) command

hmmscan --cpu 8 --domtblout TrinotatePFAM.out Pfam-A.hmm
trinity_assembly_NorRNA.fasta.transdecoder.pep > pfam.log
```

8. Predict signal peptides with **signalP** (40 min)

```
*make sure you have the script 'signalp' in your directory. This was downloaded
from the website and modified to increase the maximum allowed entries to 2,000,000
*update the path to where signalp is installed:

$ENV{SIGNALP} = '/panfs/panasas01/bisc/am14886/signalp';


#!/bin/bash
# request resources:
#PBS -l nodes=1:ppn=10,walltime=100:00:00

cd $PBS_O_WORKDIR

# signalp
./signalp -f short -n signalp.out trinity_assembly_NorRNA.fasta.transdecoder.pep
```

9. Predict transmembrane regions with **tmHMM** (60 min)

```
#!/bin/bash
# request resources:
#PBS -l nodes=1:ppn=8,walltime=100:00:00

cd $PBS_O_WORKDIR

# prediction of transmembrane helices in proteins

tmhmm --short < trinity_assembly_NorRNA.fasta.transdecoder.pep > tmhmm.out
```

10. Identify rRNA transcripts with **RNAMMER** (60 min)

```
#!/bin/bash
# request resources:
#PBS -l nodes=1:ppn=8,walltime=100:00:00

cd $PBS_O_WORKDIR
# identify rRNA transcripts
~/Trinotate-3.0.2/util/rnammer_support/RnammerTranscriptome.pl --transcriptome
trinity_assembly_NorRNA.fasta --path_to_rnammer
/panfs/panasas01/bisc/am14886/rnammer
```

Load results into Trinotate SQLite Database

```
usage: Trinotate <sqlite.db> <command> <input> [...]
```

Create Trinotate SQLite Database

1. Initialise the database; generate file with gene/transcript relationships

```
In /Trinotate-3.0.2/admin

~/Trinity-v2.6.5/util/support_scripts/get_Trinity_gene_to_trans_map.pl
~/trinity_assembly_NorRNA.fasta >  Trinity_blood.fasta.gene_trans_map
```

2. Load transcripts, protein sequences and gene/transcript relationships to the Trinotate sqlite database

```
Trinotate Trinotate.sqlite init --gene_trans_map Trinity_blood.fasta.gene_trans_map
--transcript_fasta ~/trinity_assembly_NorRNA.fasta --transdecoder_pep
~/trinity_assembly_NorRNA.fasta.transdecoder.pep LOAD_rnammer
trinity_assembly_NorRNA.fasta.rnammer.gff
```

3. Load BLAST homologies

3.1 Load transcript hits

```
Trinotate Trinotate.sqlite LOAD_swissprot_blastx
/panfs/panasas01/bisc/am14886/BLASTX/blastx.blood.outfmt6
```

3.2 Load protein hits

```
Trinotate Trinotate.sqlite LOAD_swissprot_blastp
/panfs/panasas01/bisc/am14886/BLASTP/blastp.blood.outfmt6
```

4. Load Pfam domain entries

```
Trinotate Trinotate.sqlite LOAD_pfam
/panfs/panasas01/bisc/am14886/TrinotatePFAM.out
```

4. Load transmembrane domains

```
Trinotate Trinotate.sqlite LOAD_tmhmm /panfs/panasas01/bisc/am14886/tmhmm.out
```

5. Load signal peptide predictions

```
Trinotate Trinotate.sqlite LOAD_signalp /panfs/panasas01/bisc/am14886/signalp.out
```

6. Load results of RNAMMER

```
Trinotate Trinotate.sqlite
LOAD_rnammer  /panfs/panasas01/bisc/am14886/trinity_assembly_NorRNA.fasta.rnammer.g
ff
```

Blast searches against Uniref90 database

1. Get, uncompress and index Uniref90 database

```
wget
https://data.broadinstitute.org/Trinity/__deprecated_trinotate_resources/Trinotate_
v2.0_RESOURCES/uniprot_uniref90.trinotate_v2.0.pep.gz && mv
uniprot_uniref90.trinotate_v2.0.pep.gz uniprot_uniref90.trinotate.pep.gz && gunzip
uniprot_uniref90.trinotate.pep.gz && makeblastdb -in uniprot_uniref90.trinotate.pep
-dbtype prot

read more:  https://www.uniprot.org/help/uniref
```

2. Blastx of transcript nucleotide sequences against Uniref90.

```
#!/bin/bashqs
# request resources:
#PBS -l nodes=1:ppn=10,walltime=300:00:00

cd $PBS_O_WORKDIR

# search transcript nucleotides
blastx -query /panfs/panasas01/bisc/am14886/trinity_assembly_NorRNA.fasta -db
/panfs/panasas01/bisc/am14886/Uniref90/uniprot_uniref90.trinotate.pep -num_threads
32 -max_target_seqs 1 -outfmt 6 > blastx.uniref90.blood_13april.outfmt6
```

3. Blastp of transcript TransDecoder peptide sequences against Uniref90.

```
#!/bin/bash
# request resources:
#PBS -l nodes=1:ppn=10,walltime=300:00:00

cd $PBS_O_WORKDIR

# search transcript peptides
blastp -query /panfs/panasas01/bisc/am14886/trinity_assembly_NorRNA.fasta -db
/panfs/panasas01/bisc/am14886/Uniref90/uniprot_uniref90.trinotate.pep -num_threads
32 -max_target_seqs 1 -outfmt 6 > blastp.uniref90.blood_13april.outfmt6
```

4. Load Blast results from Uniref90:

```
in ~/Trinotate-3.0.2/admin

Trinotate Trinotate.sqlite
LOAD_trembl_blastp  blastp.uniref90.blood_13april.outfmt6
Trinotate Trinotate.sqlite
LOAD_trembl_blastx  blastx.uniref90.blood_13april.outfmt6
```

5. Create output of annotation report table; with a maximum E-value of 0.00001 for reporting best blast hit and associated annotations:

```
#!/bin/bash
# request resources:
#PBS -l nodes=1:ppn=8,walltime=03:00:00

cd $PBS_O_WORKDIR
Trinotate Trinotate.sqlite report -E 0.00001 --incl_pep --incl_trans >
trinotate_annotation_report_blood_12april.xls
```

Chapter 5. General Discussion

5.1 From genes to phenotypes

Migratory species often exhibit strikingly different scales and patterns of movement (Liedvogel et al., 2011), with some populations of the same species exhibiting partial migration. Partial migration and migratory flexibility are common strategies used by several species of bats (Fleming and Eby, 2003). Migration evolved independently in several bats most likely as a need to track seasonal resources or seek adequate roosting sites (Bisson et al., 2009). In the northern Tequila bat, migration in bats usually involves moving to higher latitudes in the spring and returning to lower latitudes in the autumn, with bats very often showing fidelity to their roosts (Altringham, 2011). Strong female philopatry is common to many social mammals and has important consequences on the kinship structure of groups which, in turn, affects forms of social relationships between females (Clutton-Brock and Lukas, 2012). Most of the bat species studied to date exhibit female philopatry, which in many cases, leads to the formation of highly related matrilineal kin (Flanders et al., 2016; Rossiter et al., 2005). Following from this overview, my analyses of mtDNA demonstrate that the northern and southern populations of *L. yerbabuenae* belong to the same species and maintain matrilineal gene flow, supporting the hypothesis that long-distant migratory populations have less genetically structured populations due to mating outside of breeding areas and long-distance movements. The most common studies used to infer the spatial dynamics of bats include population genetic studies based on mitochondrial and nuclear markers (see: Rasit Bilgin, 2012; Sovic et al., 2016; Vonhof & Russell, 2015). Migratory species have shown a tendency to have less genetically structured populations over large geographical areas caused by long-distance movements of populations and mating outside breeding areas (Moussy et al., 2013). However, despite the general trend in genetic population structure of migratory populations, migratory species are also affected by factors

including dispersal capabilities, behaviour, and ecological and geographical barriers that contribute to contrasting phenotypes (Chapman et al., 2011; Moussy et al., 2013). This led to the question of to what extent is the migratory behaviour in *L. yerbabuenae* genetically determined?

Migration involves a set of complex physiological, behavioural, and morphological mechanisms (Alerstam et al. 2003; McGuire et al. 2013) Physiological mechanisms control the timing, locomotion patterns, and synchronicity of migration (McGuire et al., 2013). Migratory behaviour has evolved rapidly in many taxa at different times, can appear and disappear within a few generations (Rappole and Tipton, 1992; Mcguire, 2012). The patterns of migration are continually being modified depending on changes in the environment, for example, many species of migratory birds are known to have changed their migratory route due to variation in temperature caused by climate change (Newton, 2007); similarly, some species of salmon are migrating and reproducing earlier (Kovach et al., 2012). Furthermore, even genetically identical organisms may evolve different morphologies based on the environment in which they live, as a result of genes that evolved to be sensitive to variations in the environment and that determine an individual's fitness (Bjorklund 2006). This variation in morphology caused by differences in the environment can even be used to monitor populations of birds based on the size of the feathers and the weight of the animals (Tellería et al., 2013). Ecomorphology has also been useful to predict habitat and diet preferences of coral reef fish based on their body shape (Wainwright and Bellwood, 2002).

Studies on wing shape variation of sedentary and migratory birds, of the same species, have shown how relationships between the environment and their different natural histories

determines wing morphologies, for example, research on several species of shorebirds shows that wing shape is a more important predictor of migratory behaviour than wing length (Copete et al., 1999; Minias et al., 2015). Similarly, several studies have shown how different species of bats with different migratory strategies have morphologies better adapted for long-distance flight (see: Norberg & Rayner 1987; Schmieder et al. 2015).

As seen in chapter 3, differences in the wing morphology of the migratory population of *L. yerbabuenae* can be explained by adaptations to sustain longer distance flights, such as higher aspect ratio, higher wing loading, shorter wingspan and more pointed wings, compared with non-migratory conspecifics, and this more aerodynamic shape can potentially improve flight performance, allowing migrants to cover long distances in relatively less time. In contrast, the non-migratory populations (male and female) show adaptations to living in more cluttered environments, such as higher wingspan, lower aspect ratio and wing loading and more rounded tips, and have wings that enable them to manoeuvre inside the vegetation and to hover better as they approach their food.

To fully understand migration, we must consider the implications that the environment can have on the genes underlying this mechanism (epigenetic changes), and the consequences that such changes have on animals adapting to novel environments (Roth, 2014). Migration is but one component of an extensive suite of functionally connected traits that together form a migratory syndrome (Piersma et al., 2005). A migratory tendency might arise from genes influencing the morphology and physiology of an individual, creating a framework upon which natural selection can act (Liedvogel et al., 2011). In this study, I found evidence of adaptations to migration at the morphological level, but little evidence of population structuring; which

led me to hypothesise that the mechanisms governing migratory behaviour must be under genetic control but also influenced by the environment.

The results of the differential gene expression analyses of brain and blood transcriptomes (chapter 4) allowed me to identify the genes and pathways more likely comprising the migratory gene package in *L. yerbabuenae.* The comparison of gene expression profiles showed that migratory bats exhibit improved learning, memory and cognition as a result of the modulation of synaptic connectivity and neuron firing. Synaptic connections between neurons are modified by learning and are typical in organisms encountering novel and enriched environments (McKenzie et al., 2013; Markham and Greenough, 2004). Migrations are inherently challenging; individuals have to move great distances and across complex landscapes. Failed migration can lead to natural selection quickly altering populations and changing phenotypes rapidly (Dingle and Drake, 2007). This high potential for cognitive plasticity and for exploration behaviour could have paved the way for the evolution of migration. Future studies on positive selection on genes associated with cognition and learning across a wide range of migratory and non-migratory species in a phylogenetic context would further clarify if the same genes found in my study have also shaped the evolution of the migratory behaviour in other species of bats by convergent evolution.

Furthermore, my gene expression analyses show evidence on how the molecular clock connects with system-level circadian responses to migration. Circannual rhythms provide essential stimuli triggering the onset of migration (Gwinner, 1996). Circadian clock genes have been associated with migration in several taxa; for example; the Pacific salmon (O'Malley et al., 2010), the monarch butterfly (*Danaus plexippus*; Zhu et al. 2008; Zhan et al. 2011),

zooplankton (*Calanus finmarchicus*; Häfker et al. 2017), several species of birds including the barn swallow (*Hirundo rustica*; Saino et al. 2017), the carrion crow (*Corvus corone*; Jones et al. 2008), and the willow warbler (*Phylloscopus trochilus*; Lundberg et al. 2013; Boss et al. 2016). The results presented in chapter 4 contribute to the notion that the circadian clock is a central component of migratory behaviour and determining variability at single nucleotide polymorphisms in the candidate genes could better explain the association between the circadian clock and the migratory behaviour in bats.

Furthermore, the gene expression analysis of blood transcriptomes supports the idea that the immune system of the migratory population can deal with a wide diversity of viruses and bacteria via a broad range of antiviral interferons, natural killer cells and histocompatibility agents as previously discovered in the Egyptian roussette bat (*R. aegyptiacus*) (Pavlovich et al., 2018). These elements, modulate functions of the immune system and allows them to build a tolerance towards pathogens (Pavlovich et al., 2018). Moreover, higher expression of immune function genes may contribute to the explanation of how migratory species can stay healthy during their migration despite encountering a wide diversity of pathogens *en route* (Buehler et al., 2010). Future studies on the diversity of MHC complexes and immunoglobulins will better explain how migratory populations build resilience toward pathogens (Baker et al., 2010; Wynne et al., 2013). My research broadens our understanding of bat migration and opens the door to future studies that will further tease apart the genetic components behind the behaviour and will deepen our understanding of their remarkable immune system.

The advent of genomics allowed researchers to show that genes are not fixed elements with single functions, but that they are dynamically interacting with other genes and are influenced

by the environment (Ungerer et al., 2008; Bossdorf et al., 2008). The interactions between genes and the environment have dramatic effects on an organism's phenotypes and even influence their behaviour (Kundakovic and Champagne, 2015; Roth, 2014). The genetic architecture of animals is the basis not only for their body shapes but also for learning, memory and cognition, mechanisms which allow them to store information and make decisions when encountering novel environments (Bjorklund, 2006; Mouly and Sullivan, 2010; Sznajder et al., 2012). Together, genes and the environment create the phenotypic plasticity necessary to develop a particular behaviour on which then natural selection can act upon (Bossdorf et al., 2008).

Environmental cues such as photoperiod, temperature and resource availability can trigger physiological and behavioural responses in organisms to initiate migration (Shaw, 2016). Furthermore, both genetic and phenotypic plasticity has been shown to influence the phenotypes of migratory organisms and can manifest as changes in biochemistry, physiology, morphology, behaviour, or life history (Piersma 1998). One potential way in which phenotypic plasticity might be linked to variation in migratory tactics is through epigenetic regulation of gene expression (e.g. DNA methylation, histone tail modification, noncoding RNAs) (Baerwald et al., 2016). Epigenetic modification can mediate responses to environmental cues, including behaviour (Roth, 2014; Suderman et al., 2012; Kundakovic and Champagne, 2015). For example, DNA methylation has been involved in the photoperiodic *Clock* gene predicted migration and breeding phenology of long-distance migratory barn swallows (*H. rustica*) (Saino et al., 2017). Additionally, early life experiences can give rise to phenotypic differences that are reflected in development and behavioural differences (Suderman et al., 2012; Cameron, 2010). More recently, genome-scale methylation studies on rainbow trout (*O.*

mykiss) have shown evidence of a relationship between epigenetic variation and diversity in migration-related traits (Baerwald et al., 2016). Heritability of traits that are ecologically important are moderately high in a variety of organisms, and morphological traits have been shown to be heritable in several birds (Keller et al., 2001; Silva et al., 2017; Koehn et al., 2016). Migratory bats may have evolved more efficient flight morphology to facilitate migration but gene expression may have allowed migratory species to be more plastic in the face of selective environmental pressures. This plasticity may also allow migratory species to adapt to novel environmental conditions if these no longer favoured migration. Thus, residency could evolve rapidly through plasticity in gene expression, and morphological changes would be expected to occur at a much slower pace through incremental microevolutionary change.

5.2 Implications for conservation

It is imperative to understand adaptations and changes in the migratory behaviour of organisms such that it is not constrained by human activities (Moore, 2011). Climate change alters the dynamics of habitats and processes on which migration depends (Fischman, 2011; Lennox et al., 2016). Migrating species can be especially sensitive due to interactions with their environment, and though much work has focused on the effect of climate change, few studies have attempted to gather that information and apply it to how it will directly affect migratory behaviour (Moore, 2011).

To preserve biodiversity and ecosystem services, we must integrate information from several disciplines. Although we have an understanding of how adaptive mechanisms shape phenotypic traits, its molecular foundation remains mostly unexplored (Allendorf et al., 2010). The genomics era rapidly changed how we study organisms and can potentially

improve the precision of estimating parameters of conservation relevance such as effective population size, inbreeding and genetic drift (Frankham, 2010). Moreover, the introduction of transcriptome sequencing has led to significant findings in understanding local adaptation and genome function in nature (Stapley et al., 2010; Ungerer et al., 2008).

L. yerbabuenae is considered "Near Threatened" on the IUCN Red List of Endangered Species (IUCN, 2018) but was recently unlisted from the U.S Endangered Species List (2018) as a consequence of the efforts made by conservationists, governments and environmental groups in the last 15 years (Fish and Wildlife Service Federal Register, 2018). Although the current populations are considered large, the species still faces threats from habitat destruction, habitat change, and rapid urban growth (IUCN, 2018). In particular, the largest maternity roost known for the species in Chiapas (Los Laguitos) faces significant threats from disturbance and vandalism. As suggested in Chapter 4, this population may also be negatively affected by light pollution. The cave is in unprotected private land, only 1.5 km away from a fast expanding city and 5 km away from the National Park Cañon del Sumidero. Considerable efforts should be made to ensure the protection of this site. Genomic data allow the quantification of adaptive variation and is useful for the delineation of Conservation Units (Funk et al., 2012). The results from this project should be informative enough to recognise two Conservation Units: one in northern Mexico which undergoes a long-distance migration and a second non-migratory population that remains year-round in southern Mexico. These two Conservation Units should be managed separately for their peculiarities at the morphological, behavioural and genomic levels.

Organisms that perform long-distance migrations, across multiple habitats present a major challenge for conservation as they require coordinated management of habitats, migratory pathways and often several countries (Fleming and Eby, 2003; Fischman, 2011). Organisms that move between habitats often provide numerous functions including pollination, seed dispersal, and translocation of nutrients (Lundberg & Moberg 2003). Conserving animal migrations strengthens ecosystem resilience by providing sources for reorganisation and recolonization, especially after disturbance, and serves as an opportunity for species to cope with climate change (Fischman 2011). Studies of the genetic architecture of migration provide meaningful biological indicators of a species' potential to survive and serve as a solid framework to make educated decisions in conservation and management; they provide knowledge on how to better evaluate and mitigate the impacts that climate change will have on populations (Allendorf et al., 2010). Genomics provided an exciting opportunity to study *L. yerbabuenae;* this approach allowed me to address a broad range of questions relevant to its evolution and natural history, which should be taken into consideration in future management decisions to ensure its long-term conservation and that of its migratory behaviour.

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